D(—)-Mandelate dehydrogenase of the yeast *Rhodotorula graminis*

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FOR MY FAMILY

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ABBREVIATIONS

The abbreviations recommended by the Biochemical Journal in its instructions to authors [*Biochem. J.* (1989) **257**, 1-21] have been used with the following additions :

AU	Absorbance units
ATZ	anilinothiazolinone
BSA	bovine serum albumin
CBS	Centraalbureau voor Schimmelcultures, Baarn, Netherlands
CCY	Czechoslovak Collection of Yeasts, Bratislava, Czechoslovakia
DCIP	2,6-dichloroindophenol
DSM	Deutsche Sammlung von Mikroorganismen, Gottingen, FRG
DTT	dithiothreitol
IFO	Institute for Fermentation, Osaka, Japan
IJFM	Instituto Jaime Ferran de Microbiologia del Consejo Superior de Investigaciones Cientificas, Madrid, Spain
K'_m	apparent Michaelis constant
NCIB	National Collection of Industrial Bacteria, Aberdeen, UK
NCYC	National Collection of Yeast Cultures, Norwich, UK
NCPF	National Collection of Pathogenic Fungi, London, UK
OD	optical density
PMS	*N*-methylphenazonium methosulphate
PMSF	phenylmethylsulfonyl fluoride
p.s.i.	pounds per square inch
PTC	phenylthiocarbamyl
PTH	phenylthiohydantoin
r.p.m.	revolutions per minute
TEMED	*N*,*N*,*N'*,*N'*-tetramethylenediamine
TFA	trifluoroacetic acid
TP	tryptic peptide
UBC	University of British Columbia, Vancouver, Canada
V'	apparent maximum velocity

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SUMMARY

1 The yeast *Rhodotorula graminis* strain KGX 39 can grow on D(—)- or L(+)-mandelate as a sole source of carbon and energy. The first step in the dissimilation of mandelate involves stereospecific oxidation to phenylglyoxylate, and this is then degraded via benzaldehyde to benzoate. Preliminary evidence had indicated the presence of a dye-linked L(+)-mandelate dehydrogenase and an NAD^+-dependent D(—)-mandelate dehydrogenase in this organism. This thesis is concerned with the purification and characterization of the NAD^+-dependent D(—)-mandelate dehydrogenase.

2 A reliable and reproducible method for the purification of D(—)-mandelate dehydrogenase was developed, and the procedure involved chromatography on DEAE-Sephacel, Phenyl Sepharose and Matrex Gel Orange A. The procedure yielded 1-2 mg of protein from 40-50 g wet wt. of cells. The enzyme was homogeneous as judged by denaturing and non-denaturing polyacrylamide-gel electrophoresis and could be stored for several months at -20°C with negligible loss of activity.

3 D(—)-Mandelate dehydrogenase is a dimer as judged by comparison of the subunit M_r (38 000) and native M_r (77 200) values estimated by SDS-PAGE and gel filtration respectively.

4 The amino acid composition indicated the presence of only one cysteine residue per subunit. The approximate number of residues per subunit calculated from the amino acid composition and subunit M_r was 354.

5 The first 57 residues from the amino terminus were sequenced. No significant homologies were apparent when the sequence was compared with an archived database containing the sequences of over 10 000 proteins. However, the amino terminal did contain a run of 9 residues which could usefully be converted into an oligonucleotide "probe" for cloning the D(—)-mandelate dehydrogenase gene.

6 The absorption spectrum showed no evidence that the enzyme was associated with or contained bound flavin, haem or cytochrome as prosthetic group.

7 D(—)-Mandelate dehydrogenase catalyses the reversible interconversion of D(—)-mandelate and phenylglyoxylate. The assay procedures developed to measure D(—)-mandelate dehydrogenase involved monitoring the reduction of NAD^+ (forward/oxidation reaction) and the oxidation of NADH (reverse/reduction reaction) at 340 nm. The enzyme produced approximately equimolar amounts of phenylglyoxylate and NADH, indicating that the enzyme catalyses a reaction with a stoicheiometry of 1 mole of D(—)-mandelate oxidised per mole of NAD^+ reduced. The equilibrium constant, K'_{eq}, for the oxidation of D(—)-mandelate was calculated to be 1.59×10^{-11} M and thus reduction of phenylglyoxylate is thermodynamically favoured.

8 The optimum pH for the oxidation of D(—)-mandelate was 9.5 and the optimum for the reduction of phenylglyoxylate was 5.85. The pI value of the enzyme was 5.9.

9 D(—)-Mandelate dehydrogenase is an NAD(H)-dependent enzyme and transferred the pro-*R* hydrogen from the C_4 position of the dihydronicotinamide ring of NADH to phenylglyoxylate. The enzyme was unable to couple the oxidation of D(—)-mandelate to the reduction of $NADP^+$ or the reduction of phenylglyoxylate to the oxidation of NADPH. It was also unable to use DCIP or DCIP plus PMS as artificial electron acceptors.

10 D(—)-Mandelate dehydrogenase was not active with L(+)-mandelate as substrate although it was a competitive inhibitor of the enzyme. A number of ring-substituted mandelates and ring-containing mandelate analogues were substrates. The enzyme was not active with a range of aliphatic 2-hydroxy and 2-oxocarboxylic acids as substrates although they could inhibit enzymic activity.

11 D(—)-Mandelate dehydrogenase was not inhibited by a range of possible metabolic inhibitors including benzaldehyde, benzoate, succinate, glucose, ADP/Mg^{2+}, ATP/Mg^{2+} or acetyl-CoA, although the oxidation reaction was inhibited by high concentrations of phenylglyoxylate.

12 D(—)-Mandelate dehydrogenase was neither activated nor inhibited by a range of salts and metal ions, neither was it inhibited by the metal chelating agents EDTA, diethyldithiocarbamic acid, bathophenanthroline disulphonic acid, dihydroxybenzene disulphonic acid, pyrazole, 8-hydroxyquinilone or 2,2′-dipyridyl.

13 D(—)-Mandelate dehydrogenase was inhibited by *N*-ethylmaleimide but not by the thiol reagents 4-(chloromercuri)benzoate, iodoacetate, iodoacetamide or 5,5′-dithiobis(2-nitrobenzoic acid).

14 The K_m values for D(—)-mandelate and NAD^+ at pH 9.5 were 319 μM and 71 μM respectively, and the maximum velocity was 123 μmol min^{-1} (mg protein)$^{-1}$. The apparent K_m and V values of D(—)-mandelate, NAD^+, phenylglyoxylate and NADH at pH 5.85, pH 7.0, pH 8.0 and pH 9.5 were determined. At all values of pH tested the apparent K_m values for phenylglyoxylate and NADH were significantly lower than for D(—)-mandelate and NAD^+, and the apparent maximum velocities were significantly greater in the reverse (reduction) reaction than in the forward (oxidation) reaction.

15 Methods were developed for the synthesis and purification of the affinity label D(—)-2-(bromoacetyl)mandelic acid. Mass spectrometry, ^{1}H-NMR spectroscopy, circular dichroism and elemental analysis were used to confirm the synthesis of the compound.

16 D(—)-2-(Bromoacetyl)mandelate irreversibly inactivated D(—)-mandelate dehydrogenase. The inactivation obeyed rate saturation kinetics and the substrate, D(—)-mandelate, protected the enzyme from inactivation. The enzyme was inactivated with a stoicheiometry of 1.27 moles of label bound per mole of enzyme subunit.

17 D(—)-Mandelate dehydrogenase inactivated with D(—)-2-(bromo[^{14}C]acetyl)mandelate was digested with trypsin to produce peptides for purification and sequencing. Substrate protection experiments indicated that 2 tryptic peptides were specifically labelled by the inhibitor. Both of these (TP 1 and TP 2) were purified, although only TP 1 could be purified to homogeneity. A further, non-radiolabelled, tryptic peptide (TP 3) was purified for sequencing. All 3 tryptic peptides were sequenced and although no significant homology was evident upon comparison with a database of archived sequences, TP 1 had similar sequence to regions at the carboxy termini of *Pseudomonas putida* mandelate racemase and L(+)-mandelate dehydrogenase.

18 Several mandelate-utilising yeasts were isolated from natural environments by selective enrichment. The ability of these yeasts, and other yeasts from culture collections, to grow on D,L-mandelate as a sole source of carbon and energy was tested. Extracts of those yeasts capable of growth on D,L-mandelate were assayed for mandelate dehydrogenase activity.

19 Antisera were raised in rabbits against the *R. graminis* D(—)-mandelate dehydrogenase. Anti-*R. graminis* D(—)-mandelate dehydrogenase sera cross-reacted with their homologous antigen and with 3 other yeast NAD^+-dependent mandelate dehydrogenases, but not with the mandelate dehydrogenases of *Acinetobacter calcoaceticus*, *Lactobacillus curvatus* or the D(—)-2-hydroxyisocaproate dehydrogenase of *Streptococcus faecalis*. Anti-*A. calcoaceticus* D(—)-mandelate dehydrogenase serum cross-reacted with its homologous antigen but not with any of the other mandelate dehydrogenases tested.

20 The final chapter discusses the possible evolutionary origin of the *R. graminis* D(—)-mandelate dehydrogenase and the relationships between it and other mandelate dehydrogenases and NAD^+-dependent dehydrogenases. The possible use of this enzyme as a diagnostic test for urinary 4-hydroxy-3-methoxymandelate is also discussed. The chapter concludes with possible future lines of research.

CHAPTER 1

INTRODUCTION

1.1 The comparative enzymology of prokaryotes and eukaryotes

1.1.1 Introduction

On the whole, zoologists and botanists have used morphological criteria to classify the animal and plant kingdoms, although such criteria are of limited use in classifying microorganisms such as bacteria and yeasts. Therefore, microbiologists have turned to physiological and metabolic differences amongst microorganisms to distinguish them. However, since the advent of biochemical techniques which have allowed determination of gene sequences and the properties of their respective gene products, it has been possible to look for relationships amongst organisms at the molecular level. Indeed, it was sequence analysis of bacterial 16S ribosomal RNA (rRNA) and the analogous 18S rRNA of eukaryotes that led to the important discovery that methanogenic bacteria were a group of organisms as distinct from eubacteria and eukaryotes, as eubacteria and eukaryotes were from each other (Woese & Fox, 1977). Later studies revealed that halophilic bacteria, sulphur-dependent and thermoacidophilic bacteria were also members of the archaebacteria, and that many of their biochemical properties are exclusive to this group (Fewson, 1986).

It is well established that prokaryotes (archaebacteria and eubacteria) and eukaryotes form two distinct groups of organisms which differ with respect to their cellular organisation. For example, eukaryotes uncouple transcription and translation by means of a nuclear envelope, they possess rough endoplasmic reticulum, Golgi cisternae, microtubules, microfilaments, lysosomes and other "microbodies", larger ribosomal subunits, and have linear rather than circular chromosomes (Cavalier-Smith, 1981). However, both groups have a common requirement to carry out the essential metabolic processes, and we can therefore pose the question as to whether these two distinct forms of life have evolved similar or dissimilar metabolic pathways. The fossil record is of little use in helping to answer this question, although extant genetic sequences may be regarded as "fossils" in that they represent the successful descendants of their ancestors. We are able to determine these gene sequences, compare them and so determine whether they are likely to have evolved from a common ancestor. The sequences of many thousands of genes and proteins are now known, as are the tertiary structures of over 400 proteins (Eisenberg & Hill, 1989; Thornton & Gardner, 1989). Numerous studies have compared groups of related proteins and it is widely accepted that significant sequence homology between two proteins is indicative of evolutionary relatedness. Furthermore, detailed structural studies of particular groups such as the cytochromes, globins and serine proteases have revealed that the tertiary structure is related to the primary structure of the polypeptide and, moreover, that the difference between homologous proteins is directly related to the extent of the sequence divergence (Chothia & Lesk, 1986).

1.1.2 Central metabolic pathways

There are numerous examples of enzymes from central metabolic pathways which have been purified and characterized from both prokaryotes and eukaryotes, and this offers the opportunity to compare them and to ask the question : How similar are they ?

The most extensively characterized central metabolic pathway is the glycolytic pathway, with more known about the detailed structures of the enzymes involved than about any other comparable group (Fothergill-Gilmore, 1986). Sequence data are available for virtually all of the enzymes and in three cases, phosphofructokinase, triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase, the sequences of the enzymes from prokaryotes and eukaryotes have been determined. Unfortunately, crystallographic studies have concentrated mainly on eukaryotic enzymes, although the three dimensional structures of phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* are known (Biesecker *et al.*, 1977; Artavansis-Tsakonas & Harris, 1980; Kolb *et al.*, 1980; Walker *et al.*, 1980; Evans *et al.*, 1981; Leslie & Wonacott, 1984).

The third enzyme of the glycolytic pathway, phosphofructokinase, is an allosteric enzyme that catalyses the irreversible transfer of a phosphoryl group from ATP to fructose 6-phosphate. It is subject to feedback control and is believed to play an important role in regulating flux through the pathway. The enzymes from *E. coli* and *B. stearothermophilus* are tetramers with subunit M_r values of approximately 33 000, and although the enzymes from mammals are also tetramers they have considerably larger subunit M_r values of about 85 000 (Fothergill-Gilmore, 1986). Furthermore, the enzymes differ with respect to their mode of allosteric regulation. Whilst the bacterial enzymes are activated by ADP and GDP, and inhibited by phosphoenolpyruvate, the regulation of the mammalian enzymes is more complex as they are activated by AMP, ADP and cyclic AMP and inhibited by ATP and citrate (Fersht, 1985). On the basis of these comparisons the two enzymes would seem to be sufficiently different that the possibility of common ancestry is unlikely. However, evidence for a common evolutionary origin for the two groups has come from comparison of the amino acid sequences of the enzymes from *B. stearothermophilus* and rabbit muscle. Although the rabbit enzyme is approximately twice the size of the *B. stearothermophilus* enzyme, the bacterial enzyme is homologous to both the amino and carboxy terminal halves of the rabbit enzyme, suggesting that the mammalian enzyme has evolved by duplication and fusion of a gene to yield another homologous gene with twice its original size (Figure 1.1; Poorman *et al.*, 1984; Fothergill-Gilmore, 1986). Interestingly, the amino terminal half of the rabbit enzyme is more like the bacterial enzyme (44% homology) than is the carboxy terminal half (34% homology), and it is therefore likely that the amino terminal half has been constrained during its evolution to a greater extent than the carboxy terminal half. Presumably the amino terminal half has evolved more slowly in order to retain the catalytic

properties of the enzyme, whereas the carboxy terminal half has evolved more rapidly and has thus acquired the additional allosteric properties characteristic of mammalian phosphofructokinases (Fothergill-Gilmore, 1986).

Other eubacterial and eukaryotic glycolytic enzymes for which a common evolutionary origin has been indicated by sequence comparisons are triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase. The triosephosphate isomerases from *B. stearothermophilus* and rabbit muscle are 40% homologous, although the rabbit enzyme is more closely related to other eukaryotic triosephosphate isomerases e.g. the enzymes of *Saccharomyces cerevisiae*, coelocanth and chicken are 51%, 84% and 87% homologous respectively (Fothergill-Gilmore, 1986). Similarly, the glyceraldehyde-3-phosphate dehydrogenases from *B. stearothermophilus* and *Thermus aquaticus* are 53% and 49% homologous respectively to the enzyme from pig muscle although, as above, the pig enzyme is more closely related to other eukaryotic glyceraldehyde-3-phosphate dehydrogenases e.g. the enzymes of *S. cerevisiae*, chicken and human (Jones & Harris, 1972; Fothergill-Gilmore, 1986).

The amino acid sequence of the *B. stearothermophilus* glyceraldehyde-3-phosphate dehydrogenase is also homologous with that of the lobster enzyme, with 51% of the residues identical in both sequences (Figure 1.2). Moreover, the crystal structures of the two enzymes have been determined and the overall structure of the *B. stearothermophilus* holo-enzyme (Figure 1.3) is very similar to that of the lobster enzyme (Buehner *et al.*, 1974; Biesecker *et al.*, 1977). In each case the subunits consist of two domains, the first domain containing most of the residues involved in binding NAD^+, and the second domain containing the residues involved in catalysis. Comparison of the secondary and tertiary structural features of the bacterial and lobster NAD^+-binding domains shows them to be very similar, although the catalytic domains differ. In particular, the interactions of the β-sheet regions, β_5 with β_6 and β_7 with β_8, in the bacterial catalytic domain gives rise to a modified hydrogen bonding pattern which, in turn, results in an additional salt bridge between aspartate-293 and arginine-194 (Biesecker *et al.*, 1977). Even though the catalytic domains differ in structure, the sequence homology shown in Figure 1.2 extends throughout the length of the two proteins and presumably both enzymes had a common evolutionary origin but have undergone divergence to meet the specific requirements of each organism. For example, the *B. stearothermophilus* enzyme is notably more thermostable than the lobster enzyme and presumably the additional intra-subunit interactions such as the salt bridge between aspartate-293 and arginine-194 account for this phenomenon (Amelunxen, 1967; Amelunxen *et al.*, 1970; Biesecker *et al.*, 1977).

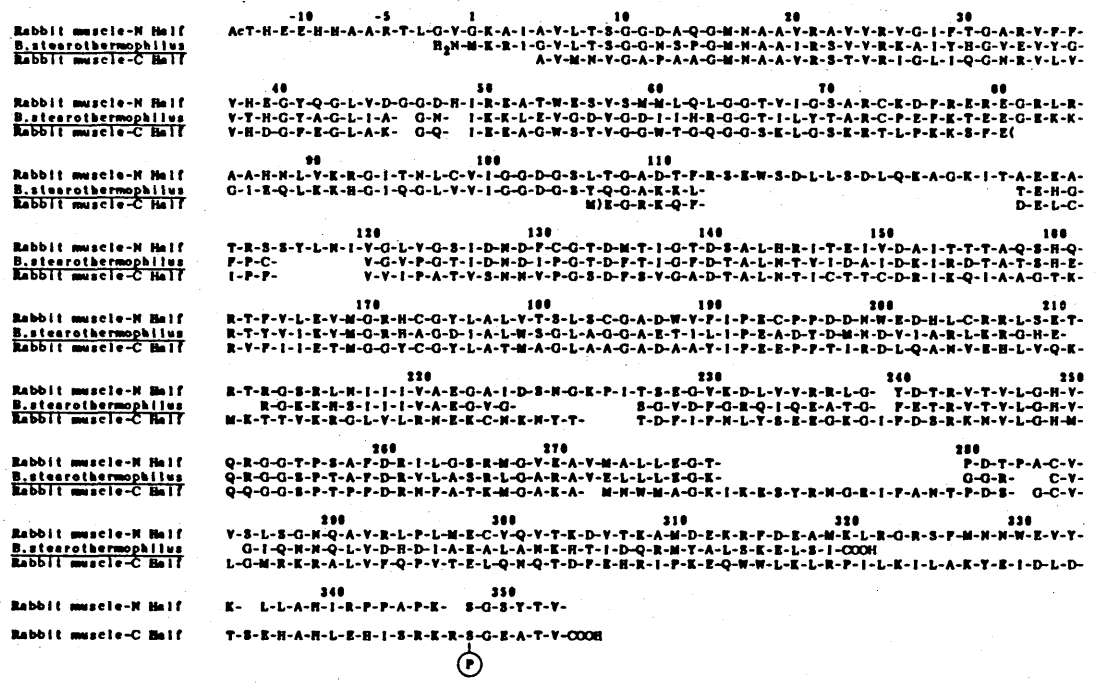


Figure 1.1 Comparison of the sequences of phosphofructokinase from rabbit muscle and *B. stearothermophilus*

The rabbit sequence is shown in two halves, with the bacterial sequence between them. The residue numbering is based on the bacterial sequence. Valine-353 in the amino terminal half of the rabbit enzyme is peptide-bonded to alanine-5 in the carboxy terminal half. Serine-348 is the site of phosphorylation. The region enclosed in parentheses (residues 81-106 of the carboxy terminal half) is of uncertain sequence. Reproduced from Poorman *et al.* (1984).

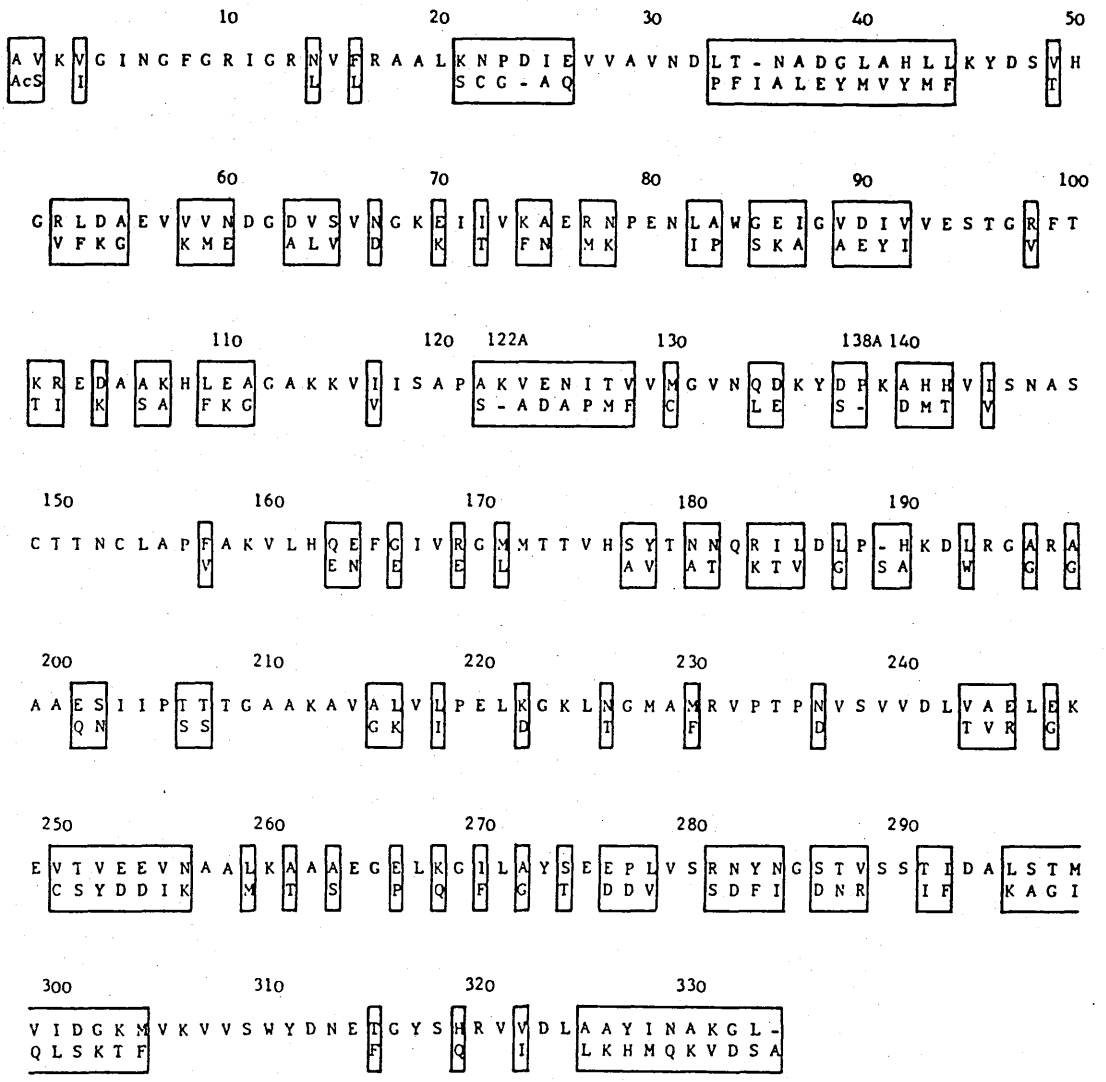


Figure 1.2 Alignment of the amino acid sequences of glyceraldehyde-3-phosphate dehydrogenase from *B. stearothermophilus* and lobster

The *B. stearothermophilus* sequence (upper) and lobster sequence (lower) are aligned to maximise homologies and regions of non-identity are boxed. Deletions are introduced at residues 35 and 189 and insertions at 122A and 138A of the bacterial enzyme. Information is taken from Davidson *et al.* (1967) and Biesecker *et al.* (1977). Reproduced from Biesecker *et al.* (1977).

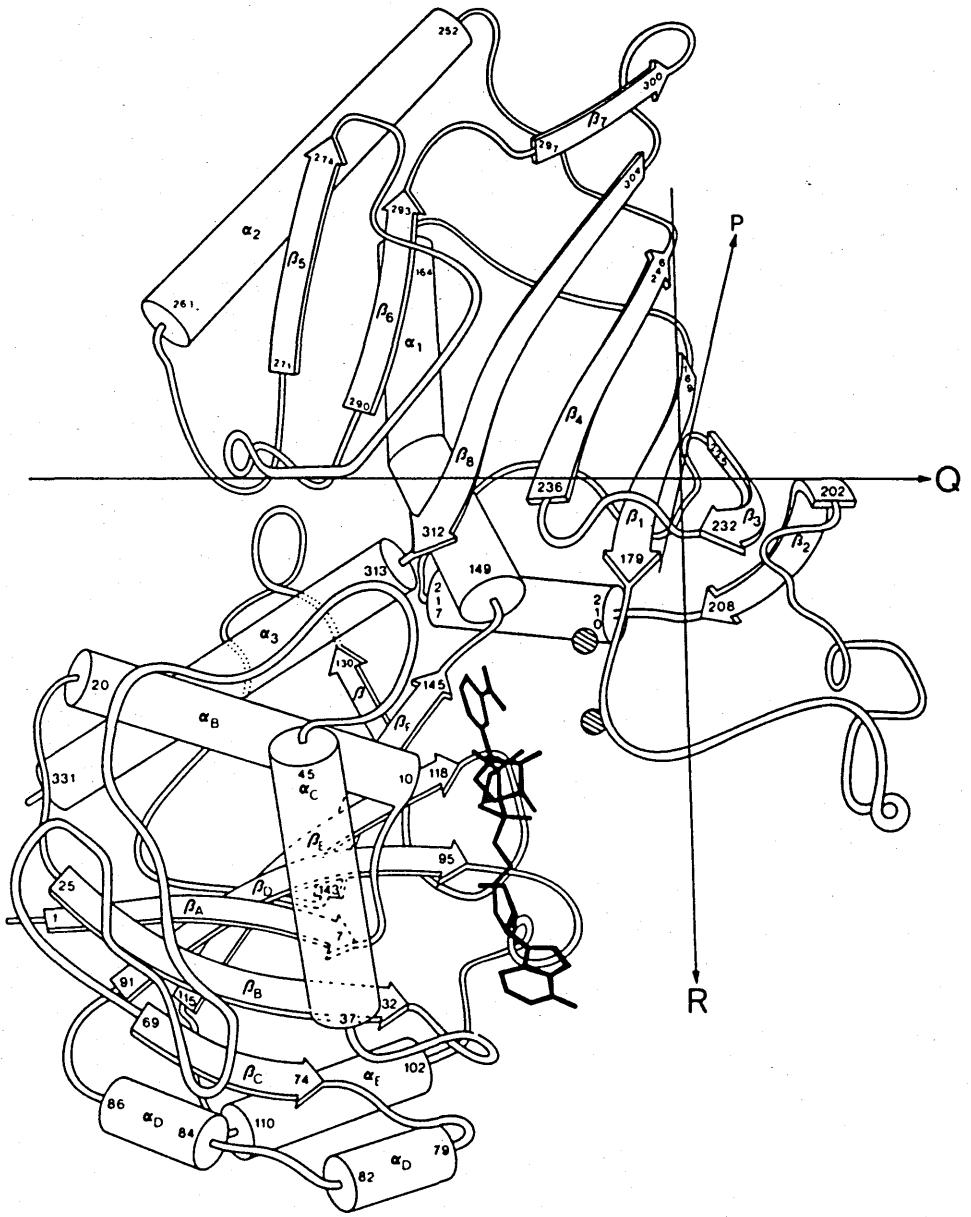


Figure 1.3 The subunit structure of holo-glyceraldehyde-3-phosphate dehydrogenase from *B. stearothermophilus*

Arrows represent β-strands, and cylinders are α-helices. A skeletal model of NAD^+ is superimposed, and the two hatched circles indicate the positions of inorganic phosphate (upper circle) and the 3-phospho group of the triose (lower circle). The secondary structure closely resembles that of lobster glyceraldehyde-3-phosphate dehydrogenase except where there are insertions or deletions in external loop regions between segments of secondary structure. Information is taken from Biesecker *et al.* (1977). Reproduced from Fothergill-Gilmore (1986).

Although such examples clearly demonstrate common evolutionary ancestry for these enzymes in eubacteria and eukaryotes, not all glycolytic enzymes show such homology. Probably the best documented case is for fructosebisphosphate aldolase, the enzyme that catalyses the reversible aldol cleavage of fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The enzymes of vertebrates, insects and higher plants are members of Class I aldolases. They are tetrameric, with the enzymes from mammals having subunit M_r values of 40 000 although the enzymes from plants are somewhat smaller and have subunit M_r values of 28 000 - 30 000 (Horecker *et al.*, 1972). During catalysis they form a Schiff-base intermediate between the carbonyl group of dihydroxyacetone phosphate and the ε-amino group of an active site lysine residue, and are inactivated by reduction with $NaBH_4$ in the presence of the substrate. In contrast, the Class II enzymes of bacteria and fungi are smaller than the Class I enzymes e.g. fructose 1,6-bisphosphate aldolase from *S. cerevisiae* is a dimer of identical 40 000 subunits (Harris *et al.*, 1969). They also differ markedly with respect to the reaction mechanism employed during catalysis. Unlike the Class I enzymes, they do not form a covalent enzyme-substrate intermediate but are dependent upon a divalent metal ion (usually Zn^{2+}) for activity (Horecker *et al.*, 1972). Therefore , it seems likely that whilst some of the glycolytic enzymes in eubacteria and eukaryotes share a common evolutionary origin, others do not, and presumably they have evolved in an independent manner.

Another enzyme of central metabolism which has been the focus of intense biochemical investigation is citrate synthase. This "housekeeping" enzyme of the TCA cycle catalyses the condensation of acetyl-CoA and oxaloacetate to form citrate, and its regulation may contribute to the overall control of the cycle. It is found in virtually all living organisms and has been characterized from a wide variety of sources including archaebacteria, Gram negative and Gram positive eubacteria, fungi, plants and animals (Weitzman & Danson, 1976). Comparison of a range of citrate synthases shows that they fall into two groups with very distinct properties; one group containing the enzymes of Gram positive eubacteria and eukaryotes, with the other containing the enzymes of the Gram negative eubacteria (Table 1.1). The two groups differ with respect to their native M_r and subunit number, although subunit M_r values are similar. They also differ with respect to inhibition by physiological concentrations of NADH and ATP, and their substrate dependence of activity i.e. allosteric versus non-allosteric modulation. On the basis of these comparisons it would seem that the two groups of enzyme evolved from quite different origins. However, comparison of the sequences of the citrate synthases from *E. coli*, *S. cerevisiae* and pig heart (Figure 1.4) show them to be conserved, and although there is less homology between the *E. coli* enzyme and either of the eukaryotic proteins, the homology is approximately 30% and is therefore significant.

The differences in physiological properties between the two groups have been shown to be related to the quaternary structure of the enzymes. Although wild-type *E. coli* normally express a citrate synthase characteristic of the Gram negative eubacteria, a

revertant mutant strain has been isolated which expresses an enzyme characteristic of the Gram positive eubacteria and eukaryotes (Table 1.2). The mutation(s) lead to a switch in phenotype, resulting in the concomitant loss of the enzyme's hexameric nature and regulatory sensitivity, and this is consistent with the view that the allosteric regulation of the wild-type *E. coli* citrate synthase by NADH and 2-oxoglutarate is dependent upon the quaternary structure (Danson *et al.*, 1979). The sequence homologies amongst the three enzymes strongly indicate common ancestry for eubacterial and eukaryotic citrate synthases, whereas the divergence of sequence between the *E. coli* enzyme and the two eukaryotic proteins reflects the different properties of the two groups. In particular, the ability of the *E. coli* enzyme to associate into tetramers and to be regulated allosterically is presumably due, in part, to the amino acid residues at the subunit interfaces i.e. the dimeric enzymes of the Gram positive eubacteria and eukaryotes as well as the mutant *E. coli* citrate synthase have different subunit interactions and therefore cannot form tetramers.

Homology amongst other eubacterial and eukaryotic TCA cycle enzymes has also been revealed by sequencing studies. Fumarase, which catalyses the reversible hydration of fumarate and L-malate, has been characterized from several sources and there appears to be two distinct classes of the enzyme. Class I contains the FUMA and FUMB enzymes of *E. coli* and the immunologically related fumarase of *Euglena gracilis*. These are characteristically thermolabile dimers with subunit M_r values of about 60 000 (Shibata *et al.*, 1985; Woods *et al.*, 1988). Both FUMA and the *E. gracilis* enzyme function in the TCA cycle under aerobic conditions, whereas FUMB is expressed under anaerobic conditions and functions to provide fumarate as an electron acceptor. Class II contains the FUMC enzyme of *E. coli* and the fumarases of *Bacillus subtilis*, *S. cerevisiae*, birds and mammals, and these are characteristically thermostable tetramers with M_r values of 48 000 - 50 000 (Beeckmans & Kanarek, 1977, 1982; Kobayashi *et al.*, 1981; Woods *et al.*, 1988). Like the fumarases of *E. coli* (FUMA) and *E. gracilis*, the Class II enzymes all seem to function in the TCA cycle. Whilst sequence comparison shows there is no obvious homology between the two classes of fumarase, there is striking homology amongst the enzymes of class II, with the *E. coli* FUMC enzyme showing 63% homology with the *B. subtilis* enzyme, 56% with the *S. cerevisiae* enzyme and 59% with the fumarase of human liver mitochondria (Miles & Guest, 1985; Woods *et al.*, 1986, 1988; Wu & Tzagoloff, 1987; Kinsella & Doonan, 1986).

Table 1.1 Properties of citrate synthases

Reproduced from Weitzman & Danson (1976).

	Gram positive eubacteria/eukaryotes	Gram negative eubacteria
Native M_r	90 000 - 100 000	240 000 - 280 000
Subunit M_r	50 000 - 60 000	50 000 - 60 000
Subunit number	2	6
NADH sensitivity	—	+
ATP sensitivity	+	—
Substrate dependence	Hyperbolic	Sigmoidal

Table 1.2 Comparison of wild-type *E. coli*, mutant *E. coli* and pig heart citrate synthase

Reproduced from Danson *et al.* (1979).

Property	Wild-type *E. coli*	Mutant *E. coli*	Pig heart
Native M_r	230 000	100 000	100 000
Substrate dependence	Sigmoidal	Hyperbolic	Hyperbolic
K_m for acetyl-CoA (μM)	400	11	7
K_m for oxaloacetate (μM)	55	10	10
Inhibition by 1 mM-2-oxoglutarate[a] (%)	75	10	14
Inhibition by 0.1 mM-NADH[b] (%)	90	0	0

[a] Determined at an oxaloacetate concentration of $2 \times K_m$

[b] Determined at an acetyl-CoA concentration of $0.5 \times K_m$

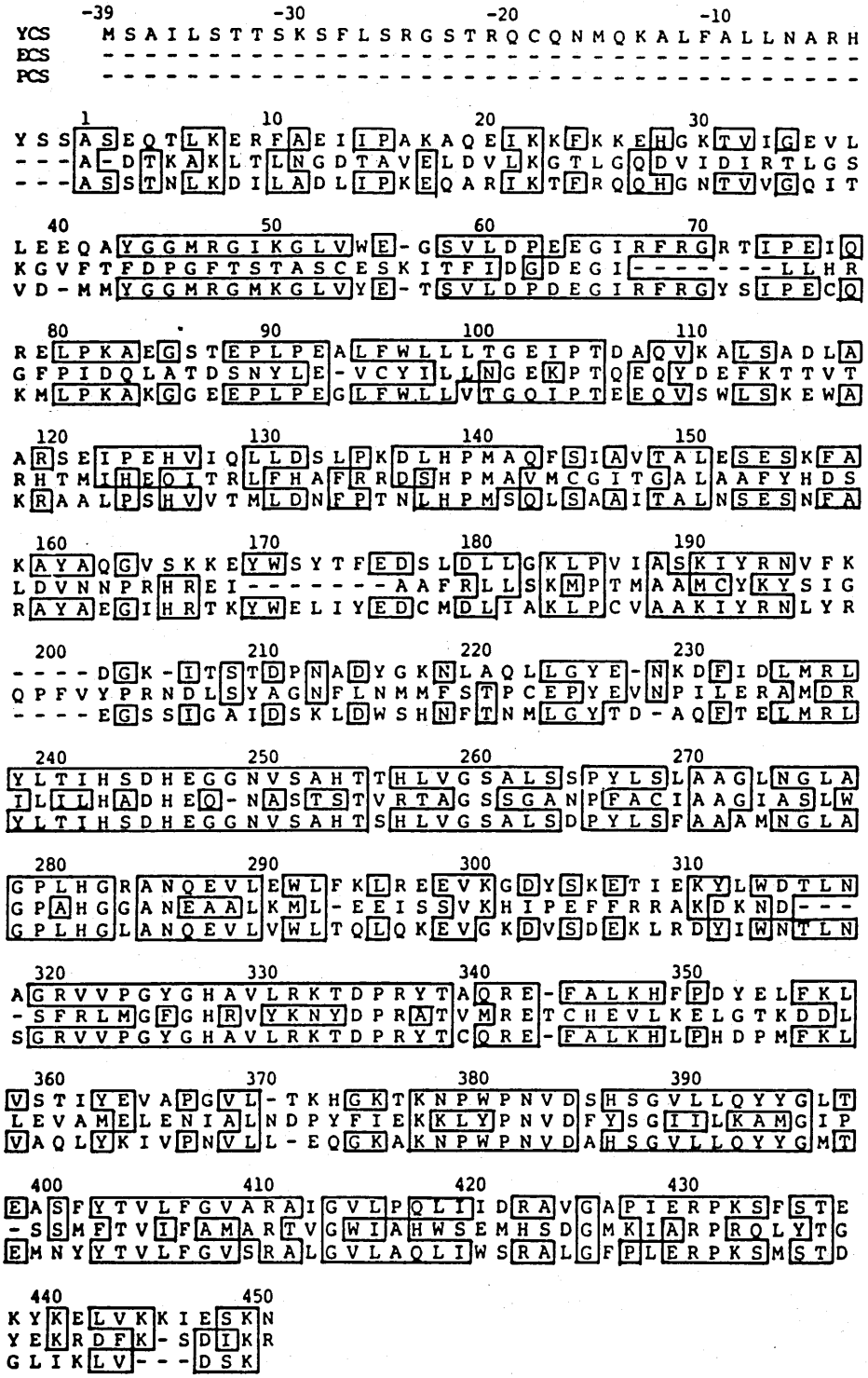


Figure 1.4 Alignment of the amino acid sequences of citrate synthase from *E. coli* (ECS), *S. cerevisiae* (YCS), and pig heart (PCS)

The sequences are aligned to maximise homologies and regions of homology are boxed. Information is taken from Bloxham *et al.* (1982), Ner *et al.* (1983), Bhayana & Duckworth (1984) and Suissa *et al.* (1984). Reproduced from Suissa *et al.* (1984).

Another central metabolic pathway which has been characterized in both prokaryotes and eukaryotes is the shikimate pathway, the biosynthetic route by which plants and microorganisms synthesize the aromatic amino acids and related compounds (Figure 1.5). Although all organisms so far studied use the shikimate pathway to convert erythrose 4-phosphate and phosphoenolpyruvate to the common precursor chorismate, there are remarkable differences amongst species in the organisation of the enzymes that catalyse the central five reactions of the pathway (Reactions 2-6 of Figure 1.5). In several prokaryotes including *Aerobacter aerogenes*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Streptomyces coelicolor* the enzymes are separable and, moreover, in *E. coli*, *B. subtilis* and *S. typhimurium* the structural genes which encode these enzymes are widely scattered about the genome (Berlyn & Giles, 1969; Henner & Hoch, 1980; Bachmann, 1983; Sanderson & Roth, 1983). However, in algae, mosses and higher plants such as *Chlamydomonas reinhardii*, *Physcomitrella patens*, *Nicotiana tabacum* and *Pisum sativum* three of the enzymes are separable but two, 3-dehydroquinase and shikimate dehydrogenase, occur as a bifunctional polypeptide (Berlyn *et al.*, 1970; Polley, 1978; Mousedale *et al.*, 1987). In fungi, the organisation is even more complex, as all five enzyme activities are found on a single polypeptide. The presence of this *arom* pentafunctional enzyme has been demonstrated in various phycomycetes, ascomycetes and basidiomycetes e.g. *Rhizopus stolonifer*, *Phycomyces nitans*, *Saccharomyces cerevisiae*, *Aspergillus nidulans*, *Neurospora crassa*, *Coprinus lagopus* and *Ustilago maydis* (Giles *et al.*, 1967; Ahmed & Giles, 1969; Lumsden & Coggins, 1977; Larimer *et al.*, 1983). Furthermore, genetic studies have revealed that the *arom* complexes of *S. cerevisiae* and *A. nidulans* are the products of single genes, indicating that these multifunctional enzymes are not formed by post-translational aggregation of five separate gene products (Charles *et al.*, 1986; Duncan *et al.*, 1987).

Although there is a marked diversity in the organisation of the five central enzymes of the shikimate pathway, comparison of the sequence of the *S. cerevisiae arom* pentafunctional enzyme with the five corresponding monofunctional enzymes of *E. coli* has revealed regions which are conserved in both the yeast and bacterial proteins (Figure 1.6) although, perhaps not surprisingly, there is a higher degree of sequence identity between the *S. cerevisiae* and *A. nidulans arom* polypeptides. Sequence analysis has also shown linker regions between the functional domains of the pentafunctional enzymes, and presumably it is these that give the *S. cerevisiae arom* polypeptide a slightly greater M_r value (174 555) than the combined M_r values of the five *E. coli* enzymes (159 698) (Duncan *et al.*, 1987). The sequence homologies between these prokaryotic and eukaryotic enzymes strongly indicate a common evolutionary origin for the shikimate pathway. Furthermore, the pentafunctional enzymes of fungi and presumably the bifunctional enzymes found in plants, are likely to have evolved by the fusion of ancestral *E. coli*-like genes (Hawkins, 1987) although, as pointed out by Hardie & Coggins (1986),

the possibility that the multifunctional enzymes are more ancient and that the monofunctional bacterial enzymes arose from them by the mutational insertion of stop and start codons, cannot be ruled out.

The ability to determine gene sequences and the properties of their respective gene products has enabled detailed comparisons of a variety of central metabolic enzymes. The sequence homologies between prokaryotic and eukaryotic enzymes of the glycolytic pathway, the TCA cycle and the shikimate pathway indicate common ancestry in the two groups of organisms, and presumably they were established early in cellular life before the prokaryotes and eukaryotes diverged. The homologies also indicate that the basic catalytic structures of the enzymes have been conserved during evolution. Indeed, many of the glycolytic enzymes seem to be evolving at a rate of only 4-6 accepted point mutations per 100 million years and aldolase (A isoenzyme) is the least rapidly changing, at 2.4 accepted point mutations over the same time scale (Fothergill-Gilmore, 1986). As the enzymes of central metabolic pathways catalyse reactions fundamental to the maintenance of life, it is perhaps not too surprising that they have evolved slowly. Selection pressures have presumably constrained the rapid evolution of these enzymes, for although mutation may well improve catalytic efficiency, rapid evolution also carries with it the increased risk of producing a reduction in efficiency or at worst, a non-functional protein.

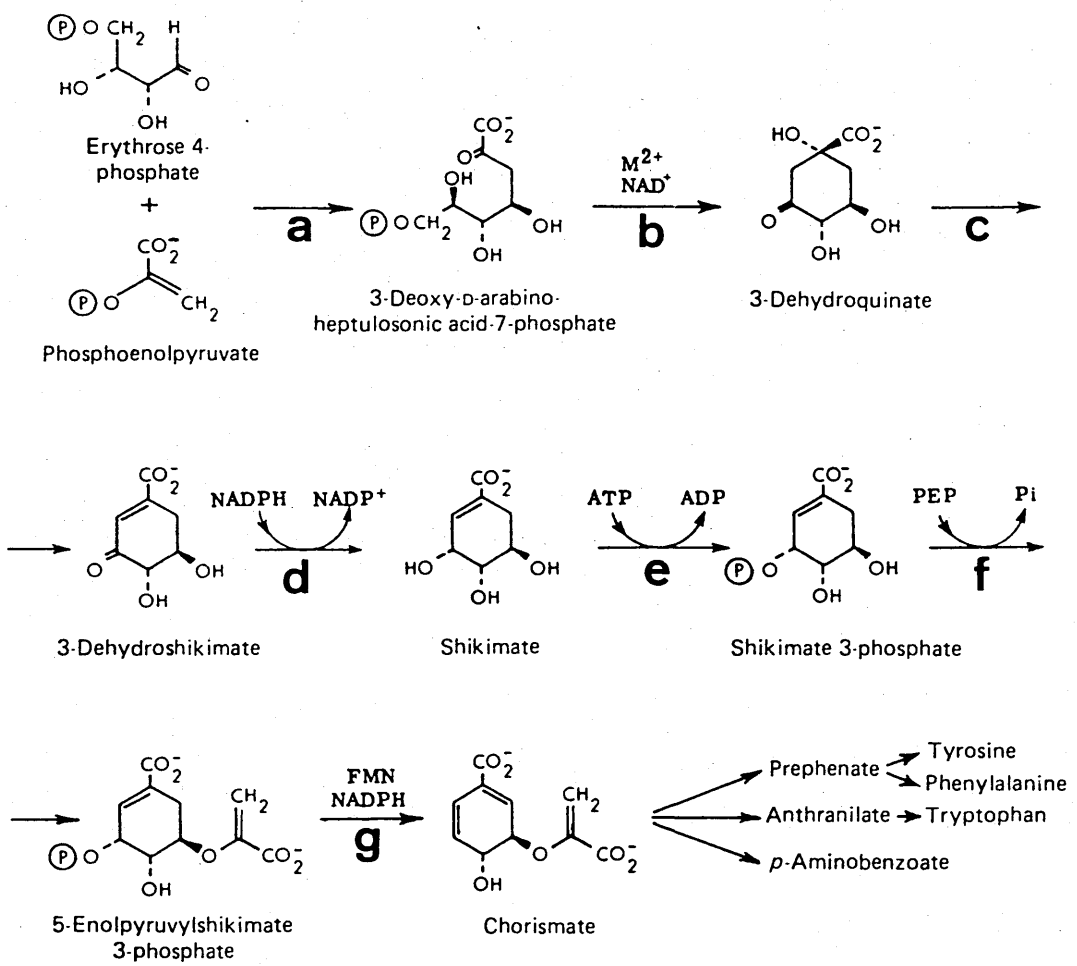


Figure 1.5 Reactions of the early common pathway of aromatic amino acid biosynthesis

Enzymes; a, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase; b, 3-dehydroquinate synthase; c, 3-dehydroquinase; d, shikimate dehydrogenase; e, shikimate kinase; f, 5-enoylpyruvylshikimate-3-phosphate synthase; g, chorismate synthase. Modified from Duncan *et al.* (1987).

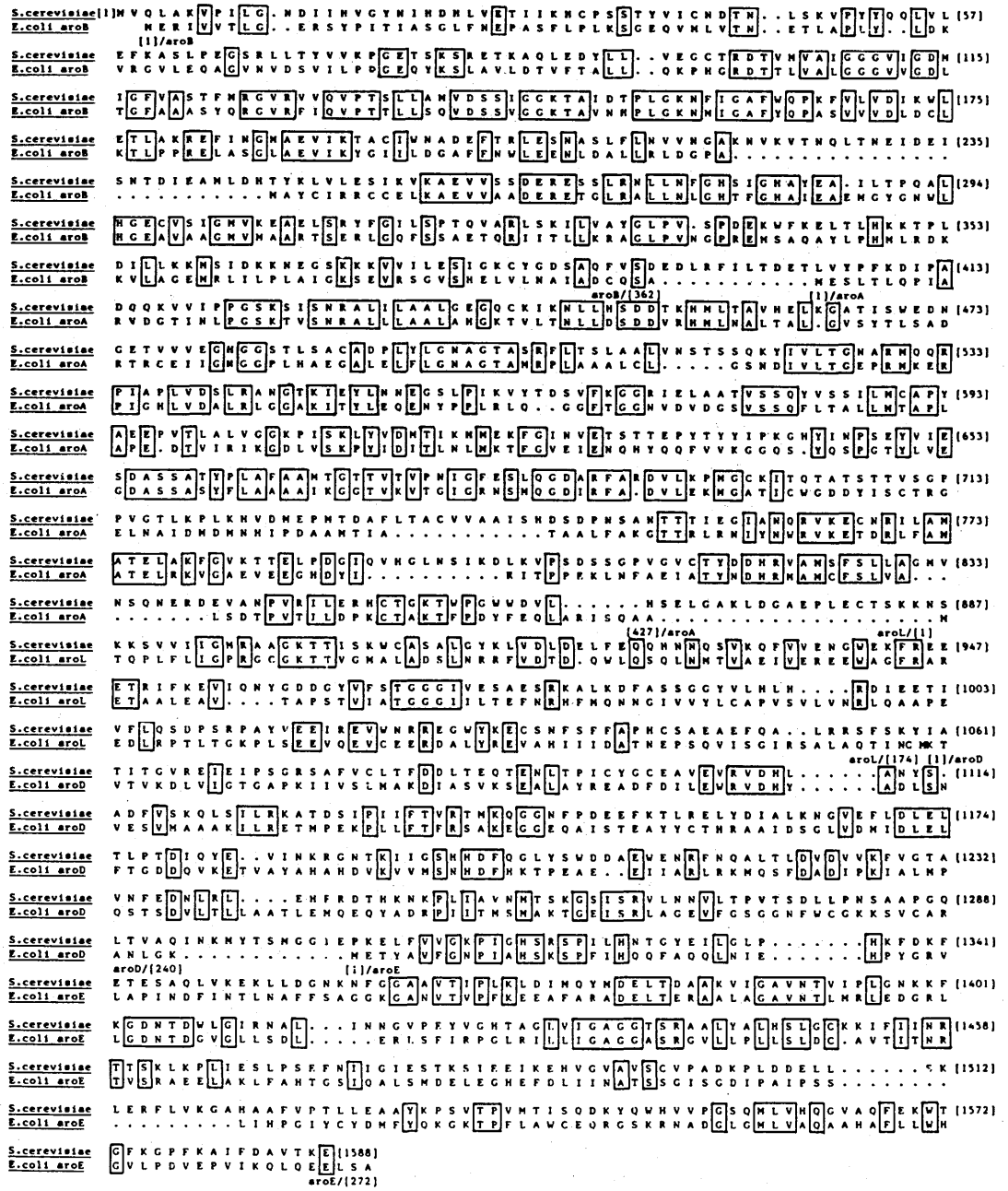


Figure 1.6 Amino acid homologies between the *S. cerevisiae arom* pentafunctional enzyme and the corresponding monofunctional *E. coli* enzymes

Enzymes; *aroB*, dehydroquinate synthase; *aroA*, 5-enoylpyruvylshikimate-3-phosphate synthase; *aroL*, shikimate kinase; *aroD*, 3-dehydroquinase; *aroE*, shikimate dehydrogenase. Numbers above and below the sequences indicate amino acid positions in the *S. cerevisiae* enzyme and in the individual *E. coli* enzymes, respectively.

Information is taken from Duncan *et al.* (1984, 1986), Millar & Coggins (1986), Millar *et al.* (1986), Duncan *et al.* (1987) and Anton & Coggins (1988). Reproduced from Duncan *et al.* (1987).

1.1.3 Peripheral metabolic pathways

Peripheral metabolic pathways enable microorganisms to metabolise a diverse range of organic compounds, and those involved in the degradation of aromatic compounds channel them to relatively few key intermediates such as catechol and protocatechuate. These, in turn, are degraded via the *ortho* and *meta*-ring cleavage pathways to yield compounds that enter the central amphibolic pathways. As aromatic compounds of natural and pollutant origin are widespread in the environment, those organisms able to utilise them gain a distinct ecological advantage over those which cannot.

Although the study of prokaryotic and eukaryotic central metabolic pathways has revealed some fascinating evolutionary relationships, the fact that the proteins are highly conserved indicates they are of ancient origin and, therefore, not best suited for investigating more recent evolutionary events. More likely candidates in this respect are the peripheral metabolic pathways. Unlike the central metabolic pathways, peripheral pathways would appear to be subject to less evolutionary constraint. Microorganisms that possess them are often nutritionally versatile, and so if mutation leads to the loss of a particular enzymic activity and, as a consequence, to the loss of the whole pathway the organism may still be able to grow on other carbon sources. Indeed, it is unlikely that microorganisms in natural environments grow on single substrates, in contrast to typical laboratory experiments. This ability to survive deleterious mutations in peripheral pathways differs from that in central pathways e.g. if one of the shikimate pathway enzymes became inactive the organism would presumably die or at least become multiply auxotrophic. Furthermore, peripheral metabolic pathways are inducible and may operate only when a particular substrate becomes available. As many of the compounds degraded via 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 accrue mutations without expression of the mutant protein. Microbial peripheral pathways may also evolve more rapidly as the chance of genetic change by transformation and/or plasmid mediated transfer are greater. Indeed, many of the enzymes of peripheral pathways are plasmid-encoded (e.g. Williams, 1981; Chalmers *et al.*, 1990*a*), 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). Despite this, most experiments aimed at unravelling evolutionary relationships amongst enzymes have concentrated on the central pathways, with relatively little known about the evolution of peripheral metabolic pathways.

However, one peripheral pathway which has been characterized in prokaryotes and eukaryotes is the quinate pathway. Several bacteria and fungi including *Acinetobacter calcoaceticus*, *Pseudomonas aureofaciens*, *Pseudomonas putida*, *Aspergillus nidulans*, *Fusarium oxysporum*, *Neurospora crassa* and *Penicillium spinulosum* can grow on quinate as a sole source of carbon and energy (Giles *et al.*, 1967; Cain *et al.*, 1968; Ahmed & Giles, 1969; Hawkins *et al.*, 1982; Van Kleef & Duine, 1988), and it is a

particularly important degradative pathway since quinate is widely distributed in plants in both the free state and as a constituent of chlorogenic acid (Pridham, 1965). The inducible quinate pathway is relatively short and consists of only three catalytic steps which convert quinate to the key intermediate protocatechuate. It has been the focus of intense study not only because of its role in recycling plant-derived quinate in the environment, but also because one of the catalytic steps; conversion of 3-dehydroquinate to 3-dehydroshikimate, is a reaction shared with the shikimate pathway (Figure 1.7).

The first enzyme of the pathway, quinate dehydrogenase, is an NAD^+-dependent enzyme in *Aerobacter aerogenes*, *P. aeruginosa* and *N. crassa*, whereas in *A. calcoaceticus* and *P. putida* the enzymes are dye-linked and dependent upon pyrrolo-quinilone quinone (PQQ) for activity (Mitsuhashi & Davis, 1954; Ahmed & Giles, 1969; Tresguerres *et al.*, 1970; Ingledew & Tai, 1972; Van Kleef & Duine, 1988). These observations demonstrate that there are two classes of quinate dehydrogenase in both prokaryotes and eukaryotes, and presumably they have evolved from independent origins. Interestingly, *P. aureofaciens*, unlike most microorganisms, contains both NAD^+-dependent and PQQ-dependent quinate dehydrogenases, although the physiological significance of this is as yet unknown (Van Kleef & Duine, 1988).

Even more fascinating evolutionary relationships have been revealed by comparison of the *A. nidulans* and *N. crassa* quinate dehydrogenases with the shikimate dehydrogenase domain of the *A. nidulans arom* polypeptide, and the product of the *N. crassa QA*-1S repressor gene. Although the proteins have quite different physiological functions i.e. the quinate dehydrogenases catalyse the oxidation of quinate and shikimate to 3-dehydroquinate and 3-dehydroshikimate respectively, shikimate dehydrogenase catalyses the reduction of dehydroshikimate to shikimate and the repressor protein regulates the expression of the quinate pathway enzymes, they nevertheless have conserved sequence (Figure 1.8; Hawkins *et al.*, 1988). The sequence homologies strongly suggest common evolutionary ancestry for these proteins and, moreover, it is likely that the catabolic quinate dehydrogenases have evolved from the anabolic shikimate dehydrogenase, and that the ability of the repressor protein to bind quinate may have evolved by acquisition of the quinate binding function from quinate dehydrogenase. If these suppositions are correct, it implies an economy in the evolution of peripheral metabolic pathways, in that organisms use pre-existing genetic information for the production of novel phenotypic traits.

The second enzyme of the pathway, 3-dehydroquinase, has been extensively studied in *A. nidulans* and *N. crassa*. The enzymes of both organisms are induced by quinate and are heat-stable, multimeric proteins (probably dodecamers) consisting of subunits with M_r values of 16 000 - 18 000. Furthermore, both enzymes show significant sequence identity with each other and antibodies raised against the *N. crassa* enzyme cross-react with the enzyme from *A. nidulans* (Hawkins *et al.*, 1982; Giles *et al.*, 1985; Da Silva *et al.*, 1986). By contrast, the *A. nidulans* and *N. crassa* biosynthetic 3-dehydroquinases are constitutively expressed, and although they form part of the *arom*

pentafunctional polypeptide, the 3-dehydroquinase domain has similar size, sequence and catalytic properties to the corresponding monofunctional *E. coli* enzyme (Chaudhuri *et al.*, 1986; Charles *et al.*, 1986; Duncan *et al.*, 1986, 1987). These observations clearly demonstrate the presence of two classes of 3-dehydroquinase in microorganisms; Class I enzymes which function in aromatic amino acid biosynthesis and Class II enzymes which function in the degradation of quinate (White *et al.*, 1990). Moreover, this division is supported by the lack of sequence homology between the biosynthetic and catabolic enzymes of *A. nidulans* (Charles *et al.*, 1985). Although this dichotomy would seem to be well defined, the constitutively expressed biosynthetic 3-dehydroquinase of *Streptomyces coelicolor*, unlike all other biosynthetic 3-dehydroquinases so far characterized, is a large multimeric protein, is notably heat-stable and its amino terminus has significant sequence homology with the catabolic enzymes of *A. nidulans* and *N. crassa* (Figure 1.9; White *et al.*, 1990). The presence of a class II "catabolic" type enzyme in the shikimate pathway of *S. coelicolor* raises some interesting questions as to the physiology and evolutionary origin of the enzyme. Although *S. coelicolor* cannot grow on quinate as a sole source of carbon and energy, and quinate dehydrogenase activity has not been detected in this organism, might it be able to co-metabolise quinate in the presence of other utilisable carbon substrates ? If this is the case, the single enzyme may serve a biosynthetic as well as a degradative function. Furthermore, what is the evolutionary relationship between this enzyme and the catabolic enzymes of *A. nidulans* and *N. crassa* and has there, at some time, been genetic transfer between the streptomycetes and filamentous fungi ?

Although few peripheral metabolic pathways have been characterized in as much detail as the quinate pathway, this example clearly shows how the study of these pathways in prokaryotes and eukaryotes can reveal evolutionary relationships which might not otherwise be revealed from examination of the more highly conserved central metabolic pathways. Moreover, if we are able to understand the processes which underlie the molecular evolution of these pathways, it would presumably aid the rational design and construction of new genes and proteins by recombinant DNA technology. Indeed, in this context many peripheral metabolic pathways are of major ecological importance as they function to re-cycle organic matter in the biosphere and, with the increasing problem of environmental pollution, attempts are being made to manipulate these pathways in order to increase the degradative capacity of microorganisms (Ramos & Timmis, 1987).

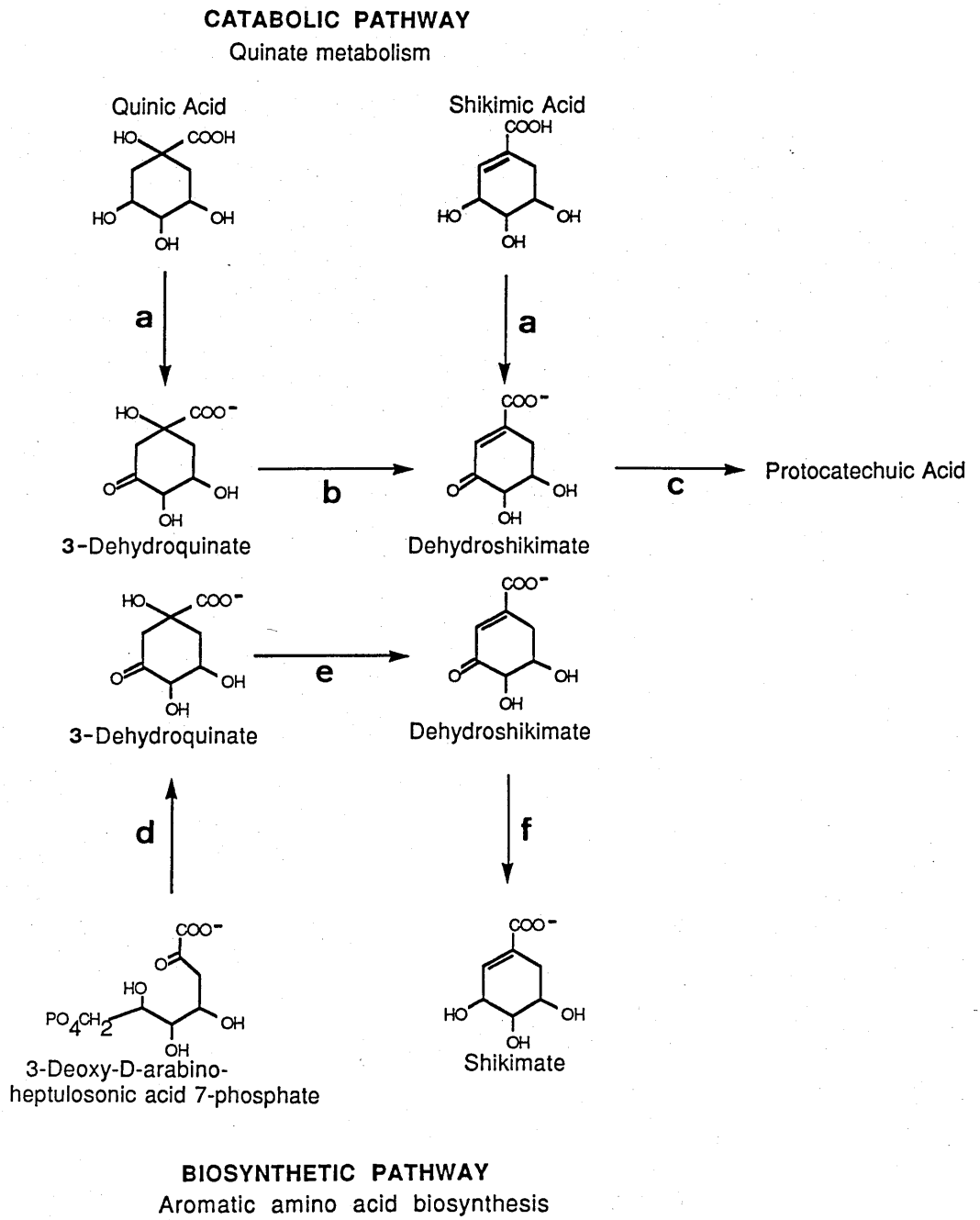


Figure 1.7 Reactions of the quinate and shikimate pathways illustrating the common enzymic conversion of 3-dehydroquinate to 3-dehydroshikimate

Enzymes; a, bi-specific quinate/shikimate dehydrogenase; b, 3-dehydroquinase; c, dehydroshikimate dehydratase; d, dehydroquinate synthase; e, 3-dehydroquinase; f, shikimate dehydrogenase.

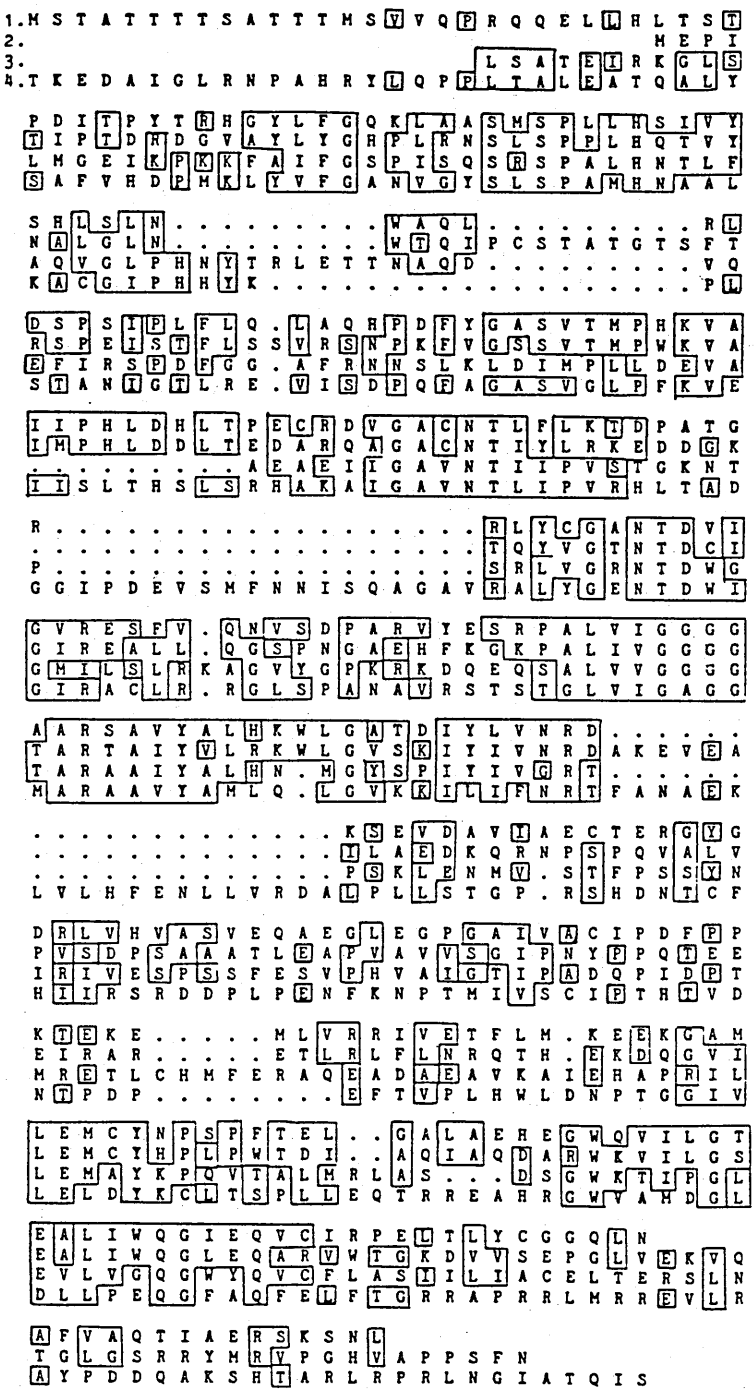


Figure 1.8 Homologies between *A. nidulans* quinate dehydrogenase, the shikimate dehydrogenase domain of the *A. nidulans arom* polypeptide, and the *N. crassa* quinate dehydrogenase and *QA*-1S repressor protein

The sequences are 1, *N. crassa* quinate dehydrogenase; 2, *A. nidulans* quinate dehydrogenase; 3, the shikimate dehydrogenase domain of the *A. nidulans arom* pentafunctional polypeptide; 4, the carboxy terminal sequence of the *N. crassa QA*-1S gene product. Gaps have been inserted into the sequence to maximise the homology which is indicated by boxing identical or closely related residues. Information is taken from Charles *et al.* (1986), Huiet & Giles (1986) and Hawkins *et al.* (1988). Reproduced from Hawkins *et al.* (1988).

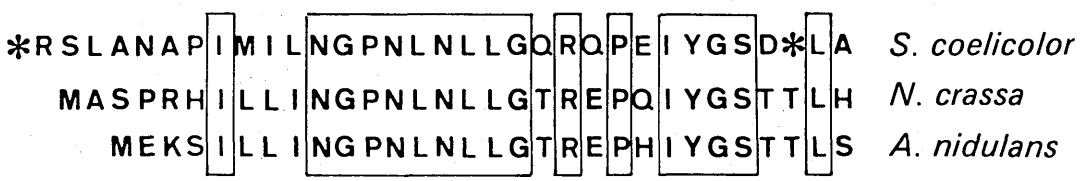


Figure 1.9 Alignment of the amino terminal sequence of the *S. coelicolor* 3-dehydroquinase with the sequence of the quinate-inducible 3-dehydroquinases from *A. nidulans* and *N. crassa*

Identical amino acid residues are boxed, and asterisks indicate residues that could not be identified unambiguously. Reproduced from White *et al.* (1990).

1.2 Microbial metabolism of mandelate

1.2.1 Mandelate in the environment

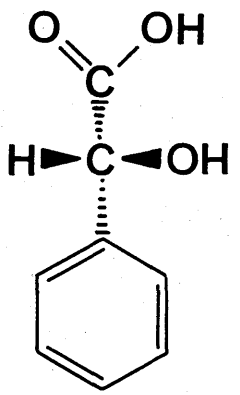
Mandelic acid (2-hydroxy-2-phenylacetic acid) is a naturally occurring aromatic 2-hydroxy acid which exists in two enantiomeric forms (Figure 1.10). The pK_a of its carboxyl group is 3.37, and so under most physiological conditions it occurs as the mandelate anion (Fewson, 1988*b*). Although there are few reports of free mandelate in nature, mandelate and a variety of related compounds continually enter the environment from several sources. For example, mandelate and phenylglyoxylate are excreted in animal urine, with the normal human urinary mandelate concentration being 1-15 μM although it may rise to elevated levels in pathological conditions such as phenylketonuria (Luthe *et al.*, 1983). Similarly, 3,4-dihydroxymandelate and 4-hydroxy-3-methoxymandelate, which are breakdown products of adrenalin and noradrenalin, are excreted in animal urine as is 4-hydroxymandelate which is a breakdown product of the neurotransmitter octopamine (Armstrong *et al.*, 1957; Axelrod & Saavedra, 1977). Urinary mandelate is also found after exposure to styrene (Chakrabarti, 1979), administration of ethylbenzene or acetophenone (Sullivan *et al.*, 1976) and after application of a solution of mandelic acid which is used as a bladder irrigation fluid to prevent urinary tract infection during urethral catheterization (Van Putten, 1979). Mandelic acid is also a component of certain drugs such as 3,3,5-trimethylcyclohexanyl mandelate, a vasoactive substance (Middleton *et al.*, 1983) and methenamine mandelate, a urinary antiseptic (Greenwood & Slack, 1981). In addition, D(—)-mandelic acid is used as a chiral synthon for the production of various semisynthetic penicillins, cephalosporins and antiobesity agents (Yamazaki & Maeda, 1986 and references therein; Vasic-Racki *et al.*, 1989) and, presumably, ingestion of such drugs leads to the excretion of mandelate and related compounds into the environment.

Mandelate and various mandelate analogues are also produced by algae, fungi and higher plants (Fewson, 1988*b*). The fungus *Aspergillus niger* produces 4-hydroxymandelate when grown on phenylalanine or phenylacetate (Kishore *et al.*, 1974, 1976), the parasitic bracket fungus *Polyporus hispidus* produces 4-hydroxymandelate from phenylalanine and tyrosine (Perrin & Towers, 1973) and there is evidence to suggest that the related fungus *Polyporus tumulosus* produces 4-hydroxymandelate, 2,5-dihydroxymandelate and 3,4-dihydroxymandelate from shikimate (Crowden, 1967). Plants are also known to produce mandelate analogues 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). Other plants synthesize glycosides of mandelonitrile e.g. (R)-prunasin and (S)-sambunigrin are the β-glucosides of D- and L-mandelonitrile respectively, and other glycosides such as (R)-holocalin, (S)-zierin, amygdalin, vicianin and lucumin contain either mandelonitrile or hydroxymandelonitrile moeities (Conn, 1981).

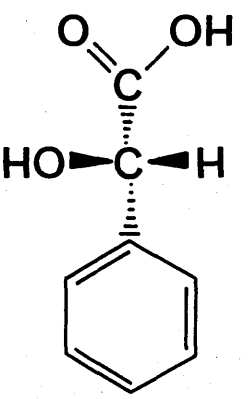
In addition to plants, some animals such as the polydesmoid millipede *Harpaphe haydeniana* synthesize and store a drop of D-mandelonitrile which it converts to benzaldehyde and HCN as a repellent (Duffey & Towers, 1978). However, as pointed out

by Fewson (1988*b*) neither the synthesis nor breakdown of mandelonitrile in plants or animals directly involves mandelate itself, although the possibility exists that liberation of these compounds into the soil might give rise to mandelate after hydrolytic reaction(s).

Although there seem to have been no attempts to quantify the amount of mandelate in nature, these examples demonstrate how mandelate and various mandelate analogues of natural and man-made origin may enter the environment and therefore serve as growth substrates for microorganisms. In some habitats mandelate and related compounds may indeed be rare, but in others they may be a permanent feature e.g. in stockyards where deposition of animal faeces and urine may provide continual supplies of 3,4-dihydroxymandelate and 4-hydroxy-3-methoxymandelate. Indeed, it was for this very reason that Sze and Dagley (1987) chose a soil sample from a cattle yard to selectively enrich an organism capable of growing on D,L-4-hydroxy-3-methoxymandelate as a sole source of carbon and energy, and it was presumably because of this that they were successful in isolating a strain of *Acinetobacter lwoffi* with the desired phenotype.



D(−)-Mandelic acid



L(+)-Mandelic acid

Figure 1.10 Enantiomers of mandelic acid

1.2.2 Bacterial metabolism of mandelate

Numerous bacteria including *Acinetobacter calcoaceticus*, *Arthrobacter* spp., *Azotobacter beijerinckii*, *Bacillus sphaericus*, *Nocardia* spp., *Pseudomonas aeruginosa*, *Pseudomonas convexa*, *Pseudomonas fluorescens*, *Pseudomonas multivorans*, *Pseudomonas putida* and *Rhizobium leguminosarum* are capable of growing on D(—)-, L(+)- or D,L-mandelate as the sole source of carbon and energy (Stanier *et al.*, 1966; Stevenson, 1967; Baumann *et al.*, 1968; Rosenberg, 1971; Bhat & Vaidyanathan, 1976*a*; Fewson, 1988*b*; Chen *et al.*, 1989). Although mandelate metabolism has been established in a diverse range of bacteria, it is only for *A. calcoaceticus* and *P. putida*, that the enzymes and regulatory processes involved have been studied in detail.

The first step in the catabolism of mandelate by *A. calcoaceticus* involves oxidation to phenylglyoxylate by a pair of stereospecific mandelate dehydrogenases which are integral membrane proteins requiring flavin as prosthetic group (Figure 1.11 b,c; Allison *et al.*, 1985*a*,*b*; Hoey *et al.*, 1987). Some strains, e.g. wild-type strain NCIB 8250, have an L(+)-mandelate dehydrogenase and can grow only on L(+)-mandelate (Kennedy & Fewson, 1968*b*), whereas other strains, e.g. wild-type strain EBF 65/65, have a D(—)-mandelate dehydrogenase and can grow only on D(—)-mandelate (Hills & Fewson, 1983*a*). However, both wild-type strains can give rise to mutants that have a second dehydrogenase, specific for the other enantiomer, that is co-ordinately regulated along with the pre-existing enzyme (Hills & Fewson, 1983*a*,*b*). Furthermore, the properties of an original D(—)-mandelate dehydrogenase (from a mutant strain derived from strain EBF 65/65) and an evolved D(—)-mandelate dehydrogenase (from a mutant strain derived from strain NCIB 8250) are indistinguishable from each other and antiserum raised against the evolved enzyme cross-reacts identically with the evolved and original enzymes (Fewson *et al.*, 1988).

The phenylglyoxylate produced from the oxidation of mandelate is then converted to benzaldehyde by a thiamin pyrophosphate-dependent phenylglyoxylate decarboxylase (Figure 1.11 d; Barrowman & Fewson, 1985), after which the benzaldehyde is oxidised to benzoate by benzaldehyde dehydrogenase I, one of a pair of NAD^+-dependent benzaldehyde dehydrogenases found in this bacterium (Figure 1.11 e; MacKintosh & Fewson, 1988*a*; Chalmers & Fewson, 1989*a*). Two isofunctional benzaldehyde dehydrogenases occur as the mandelate and benzyl alcohol pathways converge at the point of benzaldehyde, and different induction mechanisms have evolved to induce specifically the enzymes of the two pathways. During growth on mandelate, mandelate dehydrogenase, phenylglyoxylate decarboxylase and benzaldehyde dehydrogenase I are induced by phenylglyoxylate, whereas during growth on benzyl alcohol, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II are induced by benzaldehyde or benzyl alcohol (Livingstone *et al.*, 1972). The benzoate formed by oxidation of benzaldehyde is then converted to catechol, via 3,5-cyclohexadiene-1,2-diol-1-carboxylate (Figure 1.11 f,g), which is then fed into the catechol branch of the β-ketoadipate pathway, ultimately yielding the amphibolic intermediates succinate and acetyl-CoA.

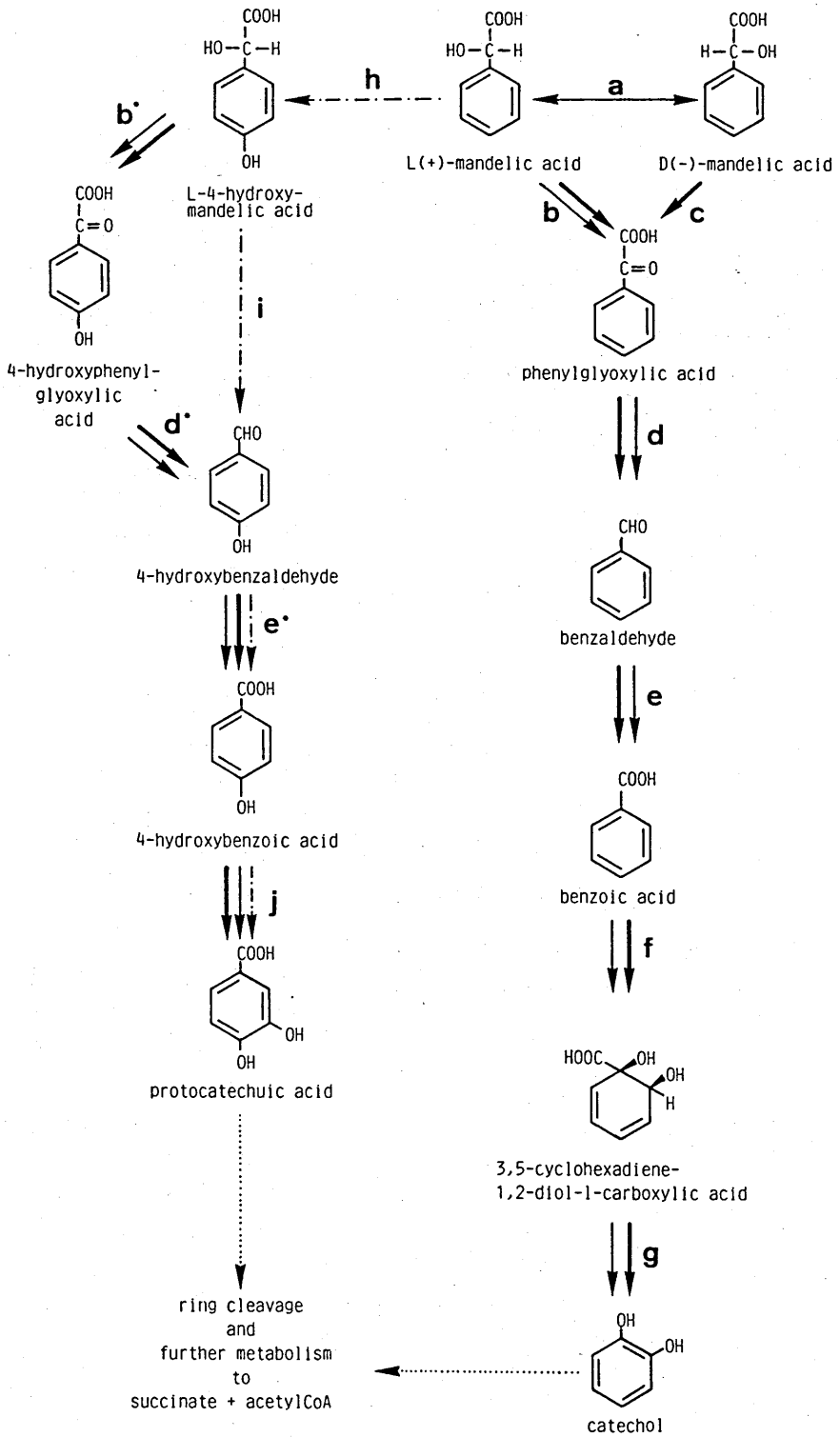


Figure 1.11 Metabolism of mandelate in *A. calcoaceticus* (⟶), *P. putida* (⟶) and *P. convexa* (⟶)

Enzymes; a, mandelate racemase; b,b*, L(+)-mandelate dehydrogenase; c, D(—)-mandelate dehydrogenase; d,d*, phenylglyoxylate decarboxylase; e,e*, benzaldehyde dehydrogenase; f, benzoate 1,2-oxygenase; g, 3,5-cyclohexadiene-1,2-diol-1-carboxylate dehydrogenase; h, L(+)-mandelate 4-hydroxylase; i, L(+)-4-hydroxymandelate oxidase; j, 4-hydroxybenzoate 3-hydroxylase. Adapted from Fewson (1988*b*).

P. putida differs from *A. calcoaceticus* in that its ability to grow on D(—)-mandelate is not endowed by the possession of a stereospecific D(—)-mandelate dehydrogenases, but by the presence of mandelate racemase and L(+)-mandelate dehydrogenase. Mandelate racemase, the most highly characterized of all the mandelate pathway enzymes, is a soluble, metal-dependent enzyme that catalyses the non-redox racemization of D(—)- and L(+)-mandelate (Figure 1.11 a; Hegeman *et al.*, 1970; Kenyon & Hegeman, 1970; Fee *et al.*, 1974*a*). L(+)-Mandelate is then converted to benzoate by the successive actions of a flavin-dependent L(+)-mandelate dehydrogenase, a thiamin pyrophosphate-dependent phenylglyoxylate decarboxylase, and two benzaldehyde dehydrogenases one of which is specific for NAD^+ and the other for $NADP^+$ (Figure 1.11 b,d,e; Hegeman, 1966*a*,*b*,*c*; Hegeman, 1970; Tsou *et al.*, 1990). Then, in a manner analogous to *A. calcoaceticus*, the benzoate formed is converted to catechol which enters the β-ketoadipate pathway to yield succinate and acetyl-CoA (Figure 1.11 f,g). However, although most strains of *P. putida* degrade mandelate by this route, a single strain has been reported which grows on both enantiomers using mandelate racemase and D(—)-mandelate dehydrogenase rather than L(+)-mandelate dehydrogenase (Kenyon & Hegeman, 1979), whereas other strains possess a D(—)-mandelate dehydrogenase in the absence of the racemase and can therefore grow only on D(—)-mandelate (Hegeman *et al.*, 1970). *P. aeruginosa* can also grow on L(+)-mandelate, which is oxidised by an L(+)-mandelate dehydrogenase with the rest of the pathway the same as that found in *P. putida* (Rosenberg, 1971).

A further difference is seen in the metabolic pathway used by *P. convexa*. This bacterium, which is probably a strain of *P. putida* (Hegeman *et al.*, 1970), has a mandelate racemase and can therefore grow on both enantiomers, but instead of oxidising L(+)-mandelate to phenylglyoxylate it hydroxylates the aromatic ring with an NADPH, tetrahydropteridine, Fe^{2+} and O_2-dependent L(+)-mandelate 4-hydroxylase to form 4-hydroxymandelate (Figure 1.11 h; Bhat & Vaidyanathan, 1976*b*). The 4-hydroxymandelate is then oxidatively decarboxylated to 4-hydroxybenzaldehyde by a membrane bound, FAD, Mn^{2+} and O_2-dependent L(+)-4-hydroxymandelate oxidase (Figure 1.11 i; Bhat & Vaidyanathan, 1976*c*). Then, after oxidation of 4-hydroxybenzaldehyde to 4-hydroxybenzoate, the 4-hydroxybenzoate is further hydroxylated at position 3 of the aromatic ring to form protocatechuate which enters the protocatechuate branch of the β-ketoadipate pathway (Figure 1.11 e*,j; Bhat & Vaidyanathan, 1976*a*). Therefore, *P. convexa* differs from all other strains of *Pseudomonas* and *Acinetobacter*, in that it hydroxylates L(+)-mandelate rather than dehydrogenating it, converts 4-hydroxymandelate to 4-hydroxybenzaldehyde without the intermediate synthesis of 4-hydroxyphenylglyoxylate and uses the protocatechuate branch of the β-ketoadipate pathway, rather than the catechol branch.

Many of the enzymes of the mandelate pathway show substrate ambiguity and will readily catalyse their respective reaction with a number of ring-substituted analogues. For example, *A. calcoaceticus* will to grow on 2-hydroxymandelate, 4-hydroxymandelate, 3,4-dihydroxymandelate and 4-hydroxy-3-methoxymandelate, degrading them to either catechol or protocatechuate (Figure 1.12; Kennedy & Fewson, 1968*b*) and, likewise, *P. putida* grows on 4-hydroxymandelate (Gunter, 1953). However, some ring-substituted mandelates e.g. 4-chloromandelate and 4-bromomandelate do not support growth of either bacterium as they are metabolised only to the level of the corresponding halo-benzoate (Hegeman, 1966*a*; Kennedy & Fewson, 1968*b*). However, as pointed out by Fewson (1988*b*), partial metabolism may nevertheless be important in nature where mixed populations of microorganisms may totally degrade compounds which are beyond the scope of any one individual microorganism.

The mandelate pathway also acts as a metabolic "funnel" by allowing growth on a variety of aromatic substrates which can be metabolised to any one of the pathway intermediates. For example, *P. putida* can convert ethylmandelate, mandelamide, methylmandelate, phenyl 1,2-ethanediol and phenylglyoxal to mandelate (Hegeman, 1966*a*), L-phenylglycine to phenylglyoxylate (Van den Tweel *et al.*, 1986) and those strains which contain the TOL plasmid can convert toluene to benzaldehyde via benzyl alcohol (Worsey & Williams, 1975) (Figure 1.13) Therefore, if an organism possessing the mandelate pathway, evolves a new enzymic activity which converts a previously non-metabolisable compound to an intermediate of the mandelate pathway it should be able to grow on an additional substrate.

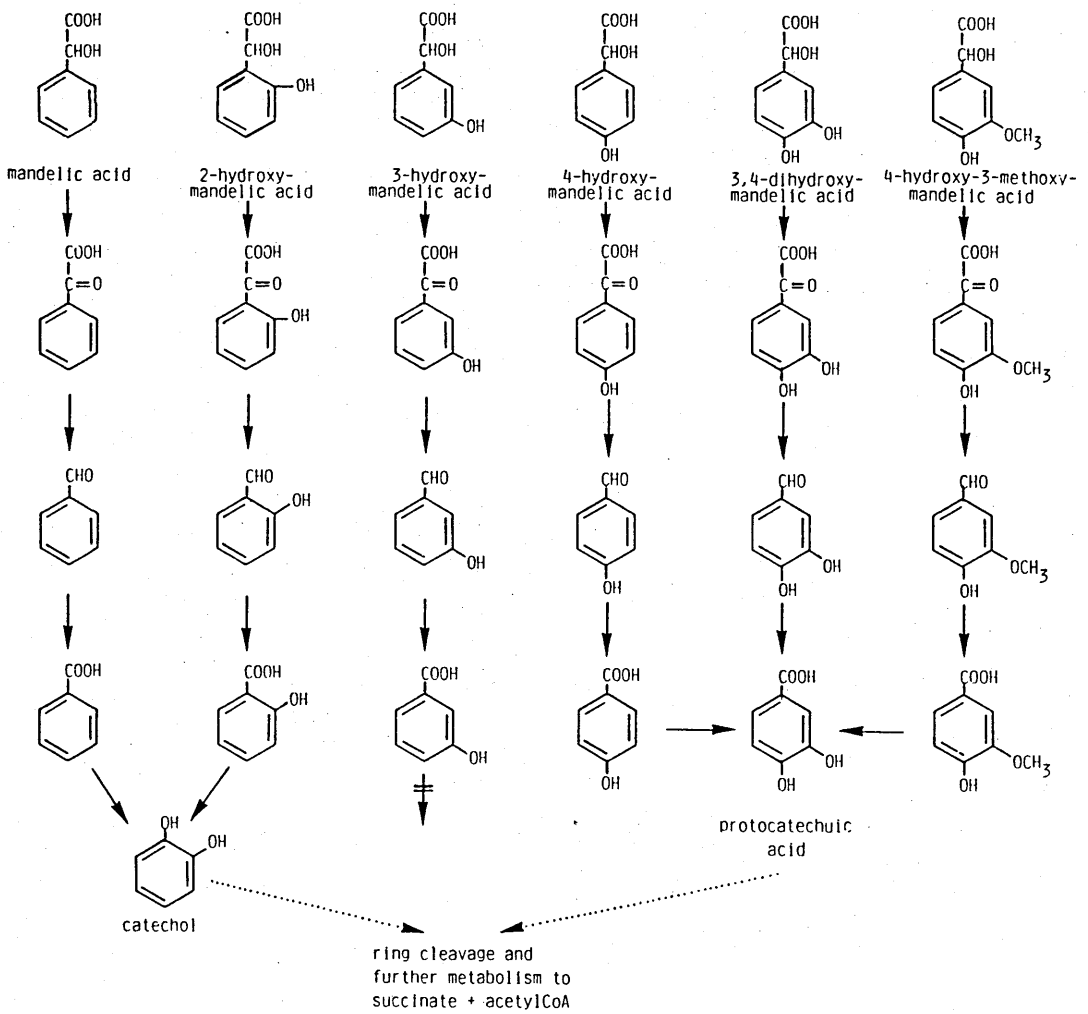


Figure 1.12 Metabolism of ring-substituted mandelates by *A. calcoaceticus*

Information is taken from Kennedy & Fewson (1968*b*). Reproduced from Fewson (1988*b*).

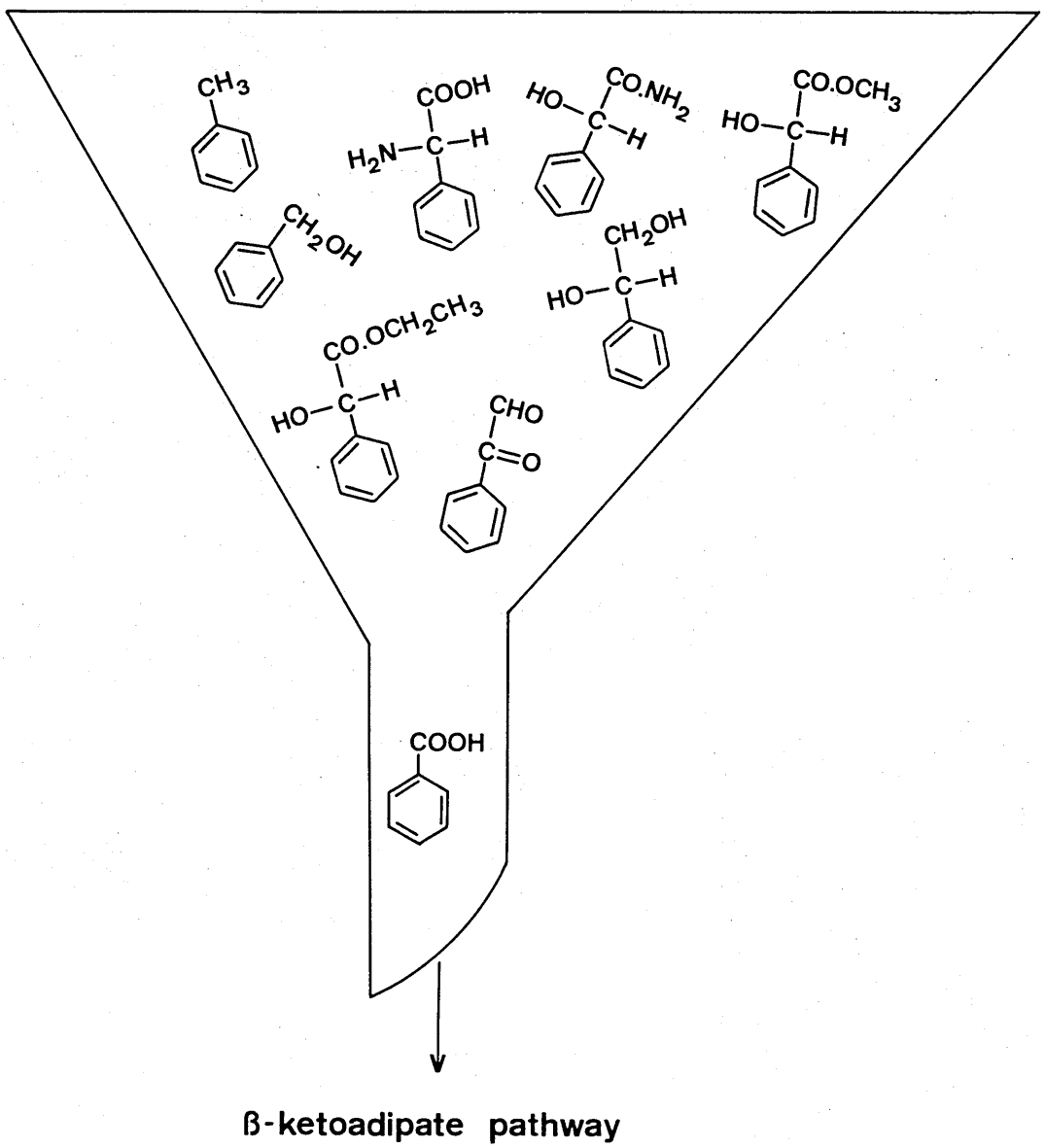


Figure 1.13 A schematic representation of the mandelate pathway of *P. putida* as a "metabolic funnel" for the dissimilation of a range of compounds to benzoate and their channeling into the β-ketoadipate pathway

Information is taken from Hegeman (1966a), Worsey & Williams (1975) & Van den Tweel *et al.* (1986). Adapted from Ramos & Timmis (1987).

1.2.3 Fungal metabolism of mandelate

Several fungi including *Aspergillus flavus*, *Byssochlamys fulva* and *Rhodotorula graminis* can grow on D(—)-, L(+)- or D,L-mandelate as the sole source of carbon and energy (Iyayi & Dart, 1980; Dart & Iyayi, 1981; Durham *et al.*, 1984), whereas other fungi e.g. *Aspergillus niger* and *Neurospora crassa*, which degrade both enantiomers in the presence of other carbon sources, may not necessarily be able to sustain growth on mandelate alone (Jamaluddin *et al.*, 1970; Ramakrishna Rao & Vaidyanathan, 1977). Metabolism of mandelate by fungi is less well understood than is bacterial mandelate metabolism and, moreover, there seem to have been no reports of any of the mandelate pathway enzymes having been purified to homogeneity from a fungal source, although some of the enzymes have been studied in crude extract or in partially purified form.

In all fungi so far examined, the first step in the dissimilation of mandelate is always oxidation to phenylglyoxylate catalysed by either D(—)- or L(+)-mandelate dehydrogenases. In *A. niger* strain UBC 814 there is a pair of stereospecific mandelate dehydrogenases; a soluble, dye-linked L(+)-mandelate dehydrogenase which is stimulated by FAD and FMN, and a "particulate" D(—)-mandelate dehydrogenase which, unlike the L(+)-mandelate dehydrogenase, cannot reduce 2,6-dichloroindophenol and is not stimulated by either of the flavins (Figure 1.14 a,b; Jamaluddin *et al.*, 1970). However, as the D(—)-mandelate dehydrogenase was assayed by estimating the amount of phenylglyoxylate formed, and the effect of nicotinamide nucleotides on its activity was not determined, it is unclear whether this enzyme is NAD(P)$^+$-dependent. In a different strain of *A. niger*, there are D(—)- and L(+)-mandelate dehydrogenases associated with both soluble and "particulate" fractions of the crude extract. The enzymes in both fractions were found to use NADP$^+$ and, to a lesser extent, NAD$^+$ as cofactors for the oxidation of D,L-4-hydroxymandelate, although the stereospecificities of the enzymes were not determined (Kishore *et al.*, 1976). There is also an NAD$^+$-dependent mandelate dehydrogenase in *B. fulva*, although as with the *A. niger* enzymes, its stereospecificity was not demonstrated (Iyayi & Dart, 1986). In the yeast *R. graminis*, there is a pair of stereospecific mandelate dehydrogenases; a membrane "associated", dye-linked L(+)-mandelate dehydrogenase and a soluble, NAD$^+$-dependent D(—)-mandelate dehydrogenase (Figure 1.14 a,b; Durham, 1984). This was the first clearly reported case of an NAD$^+$-dependent dehydrogenase being involved in the oxidation of mandelate and, as shown later in this thesis (Section 4.3.1), it catalyses the reversible interconversion of D(—)-mandelate and phenylglyoxylate. It is presumably a similar enzyme in the bracket fungus *Polyporus tumulosus* which converts 4-hydroxyphenylglyoxylate to 4-hydroxymandelate, although this observation was based upon radiolabelling studies rather than assay of the enzyme involved (Crowden, 1967).

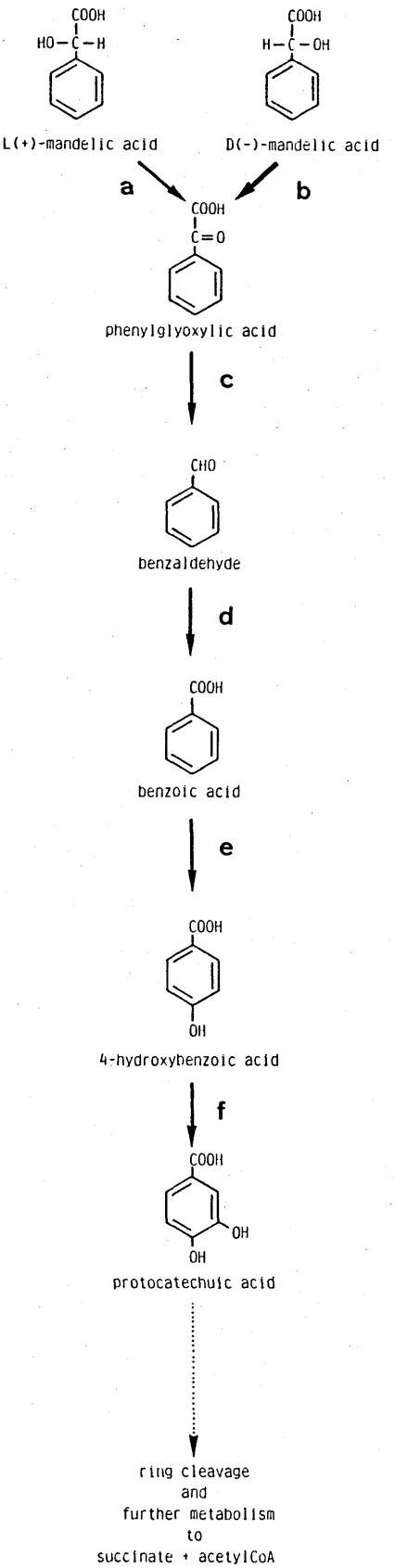


Figure 1.14 Metabolism of mandelate in *A. niger*, *N. crassa* and *R. graminis*

Enzymes; a, L(+)-mandelate dehydrogenase; b, D(—)-mandelate dehydrogenase; c, phenylglyoxylate decarboxylase; d, benzaldehyde dehydrogenase; e, benzoate 4-hydroxylase; f, 4-hydroxybenzoate 3-hydroxylase. Adapted from Fewson (1988*b*).

The phenylglyoxylate produced from the oxidation of mandelate is then converted to benzaldehyde, and in *A. niger*, *N. crassa* and *R. graminis*, as in bacteria, the enzyme is dependent upon thiamin pyrophosphate for activity (Figure 1.14 c; Jamaluddin *et al.*, 1970; Kishore *et al.*, 1976; Ramakrishna Rao & Vaidyanathan, 1977; Durham, 1984). Benzaldehyde is then oxidised to benzoate by nicotinamide nucleotide-dependent benzaldehyde dehydrogenases, and in *A. niger* strain UBC 814 there are two isoenzymes, one of which is specific for NAD^+ and the other for $NADP^+$, whereas in *R. graminis* there is a single NAD^+-dependent enzyme (Figure 1.14 d; Jamaluddin *et al.*, 1970; Durham, 1984). *R. graminis* also grows on benzyl alcohol as a sole source of carbon and energy, and converts benzyl alcohol to benzaldehyde by means of an NAD^+-dependent benzyl alcohol dehydrogenase (Middleton, 1988). However, it is not known whether the yeast has a second benzaldehyde dehydrogenase specifically associated with benzyl alcohol metabolism in addition to the NAD^+-dependent enzyme induced during growth on mandelate.

The mandelate pathway of fungi then diverges from that found in bacteria, as the benzoate formed from the oxidation of benzaldehyde is converted to 4-hydroxybenzoate rather than to 3,5-cyclohexadiene-1,2-diol-1-carboxylate (Figure 1.14 e). In *A. niger* strain UBC 814 benzoate 4-hydroxylase is a soluble, tetrahydropteridine, NADPH and O_2-dependent enzyme, whereas in *R. graminis* it is membrane "associated", pteridine-independent and probably contains flavin as a cofactor (Reddy & Vaidyanathan, 1975; M^{c}Namee & Durham, 1985). The final enzymic step prior to ring cleavage is further hydroxylation by 4-hydroxybenzoate-3-hydroxylase to form protocatechuate, and in *R. graminis* the enzyme is soluble and NADPH-dependent (Figure 1.14 f, M^{c}Namee & Durham, 1985). Protocatechuate is then fed into the protocatechuate branch of the β-ketoadipate pathway, ultimately yielding the amphibolic intermediates succinate and acetyl-CoA.

1.2.4 The evolution of the mandelate pathway

Comparison of the mandelate pathways of *A. calcoaceticus* and *P. putida* with the pathway used by fungi (Figures 1.11 & 1.14) indicates that whilst they share some common steps i.e. dehydrogenation of mandelate, decarboxylation of phenylglyoxylate and dehydrogenation of benzaldehyde, they diverge at the level of benzoate resulting in the formation of catechol in bacteria and protocatechuate in fungi. Therefore, it seems likely that these pathways have evolved independently in prokaryotes and eukaryotes, and that the pre-existing genetic material in the two groups may have dictated the direction of mandelate catabolism by the evolving organisms. However, as little is known about the metabolism of mandelate in fungi, further studies are required before detailed comparisons can be made with the analogous bacterial pathways. In bacteria, however, the pathways for the degradation of mandelate have been characterized in more detail, and this offers the opportunity to investigate the possible mechanisms by which they have evolved. Although this section refers to the mandelate pathway, much of the discussion is equally applicable to the evolution of peripheral metabolic pathways in general.

The intriguing question is : How did the mandelate pathway evolve ? Horowitz (1945) proposed a mechanism of "retrograde evolution" to account for the early evolution of biosynthetic pathways and this idea could, in principle, be applied to catabolic pathways. He suggested that the substrate-binding site of an enzyme might serve as the product-binding site in a homologous enzyme, which after acquisition of a novel catalytic site by mutation, could mediate the preceding step in the evolving pathway. For example, let us assume that the ancestor of the modern day *A. calcoaceticus* strain NCIB 8250 could grow on benzoate as a sole source of carbon and energy, but not on benzaldehyde, phenylglyoxylate or mandelate. By the mechanism proposed by Horowitz, duplication and mutation of the benzoate 1,2-oxygenase gene, so that the new gene product could bind benzaldehyde and dehydrogenate it to benzoate, would extend the pathway and enable the organism to grow on an additional carbon source. Subsequent duplication and mutation of the new benzaldehyde dehydrogenase gene could give rise to phenylglyoxylate decarboxylase, and so on to give mandelate dehydrogenase.

However, comparison of the enzymes that catalyse the successive steps of the mandelate pathway in *A. calcoaceticus* and *P. putida* show them to have markedly different properties e.g. mandelate racemase is soluble and metal-dependent, the mandelate dehydrogenases are flavin-dependent, integral membrane proteins, the phenylglyoxylate decarboxylases are thiamin pyrophosphate-dependent and the benzaldehyde dehydrogenases are nicotinamide nucleotide-dependent enzymes (See Section 1.2.2 and references therein). Furthermore, several of the successive steps involve radically different chemical mechanisms. Perhaps not surprisingly, the sequences of mandelate racemase, L(+)-mandelate dehydrogenase and phenylglyoxylate decarboxylase from *P. putida* do not appear to be homologous (Tsou *et al.*, 1990). Therefore, it is difficult to imagine the mechanism proposed by Horowitz having given rise to the mandelate pathway, especially as major mutational restructuring of the gene

products would have been required at each step i.e. to alter the cofactor specificity, reaction mechanism and in one case to enable the enzyme to assume a completely different intracellular location. Similarly, retrograde evolution does not seem to have had a major role in the evolution of the glycolytic pathway as the structures and properties of the enzymes that catalyse consecutive reactions are, by and large, quite dissimilar (Fothergill-Gilmore, 1986). Nevertheless, it is unlikely that retrograde evolution has played no part at all in the evolution of metabolic pathways since there is extensive homology between the *met*B and *met*C genes which code for consecutive enzymes in the methionine biosynthetic pathway of *E. coli* (Parsot *et al.*, 1987).

A more likely explanation to account for the evolution of the mandelate pathway, and in view of the fact that economy in biological systems is an obvious selective advantage, is that the enzymes of the pathway were recruited from other metabolic pathways. Indeed, the *P. putida* L(+)-mandelate dehydrogenase sequence is homologous to other 2-hydroxyacid dehydrogenases e.g. the amino terminal has sequence identity with spinach glycolate oxidase, as does its carboxy terminal with the carboxy termini of *S. cerevisiae* glycolate oxidase and cytochrome b_2 lactate dehydrogenase (Guiard, 1985; Volokita & Somerville, 1987; Cederlund *et al.*, 1988; Tsou *et al.*, 1990). Similarly, phenylglyoxylate decarboxylase shows several regions of significant sequence homology with other thiamin pyrophosphate-dependent decarboxylases, namely *E. coli* pyruvate oxidase, the pyruvate decarboxylases from *S. cerevisiae* and *Zymomonas mobilis* and the acetolactate synthases 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). The similarities between L(+)-mandelate dehydrogenase and other FMN-dependent 2-hydroxyacid dehydrogenases and between phenylglyoxylate decarboxylase and other thiamin pyrophosphate-dependent decarboxylases strongly favours the suggestion that both these enzymes have evolved by recruitment of enzymes from other metabolic pathways already established in *P. putida* (Tsou *et al.*, 1990). Evidence to support this notion has come from the observation that the primary, secondary and tertiary structures of *P. putida* mandelate racemase and *cic*,*cis*-muconate lactonizing enzyme are homologous. Both enzymes form α/β barrels, characteristic of triosephosphate isomerase, and are superimposable on one another. Furthermore, the two enzymes have identical quaternary structures and several catalytically important residues in mandelate racemase are conserved in the lactonizing enzyme e.g. the three residues in the racemase active site which coordinate the Mg^{2+} ion (Tsou *et al.*, 1990; G. A. Petsko, personal communication).

Although evidence is coming to light which indicates that the mandelate pathway has probably arisen by means of recruitment of enzymes from other metabolic pathways, concomitant with this acquisition must have been mechanisms which put them under appropriate regulatory control. Inability to express the newly evolved enzymes in the presence of the substrate would carry with it no selective advantage, and constitutive expression in the absence of the substrate would be wasteful. In *P. putida* the genes

which encode mandelate racemase (*mdl*A), L(+)-mandelate dehydrogenase (*mdl*B) and phenylglyoxylate decarboxylase (*mdl*C) are arranged in an operon (*mdl*CBA), whilst the genes encoding the NAD^+ and $NADP^+$-dependent benzaldehyde dehydrogenases (*mdl*D and *mdl*E respectively) are clustered approximately 2 kilobases upstream. However, despite this spatial separation all 5 enzymes are co-ordinately induced by D(—)-mandelate, L(+)-mandelate or phenylglyoxylate (Hegeman, 1966*b*,*c*; Tsou *et al.*, 1990). In contrast, the mandelate enzymes of *A. calcoaceticus* do not appear to be clustered in the same manner and, moreover, they are co-ordinately induced by phenylglyoxylate and not by either enantiomer of mandelate (Livingstone & Fewson, 1972; Vakeria *et al.*, 1984, 1985). Therefore, even though the mandelate enzymes of *A. calcoaceticus* and *P. putida* have very similar properties, they are subject to quite different regulation which suggests that evolutionary constraints on catalytic function and regulation are presumably not the same (Fewson, 1988*b*).

In order to understand more fully the enzymology of mandelate metabolism and to address questions regarding the evolution of the pathway in fungi, *R. graminis* was chosen for further study. It seemed the most promising eukaryote to investigate, not only because it was a yeast and would therefore be easier to grow than filamentous fungi but also because of the high specific activities of the enzymes involved (Durham, 1984; Durham *et al.*, 1984).

1.3 *Rhodotorula graminis* and the genus *Rhodotorula*

According to Fell *et al.* (1984), the genus *Rhodotorula* contains eight recognised species : *Rhodotorula acheniorum*, *R. araucariae*, *R. aurantiaca*, *R. glutinis*, *R. graminis*, *R. lactosa*, *R. minuta* and *R. rubra*, although other species have since been isolated and proposed as members e.g. *R. grinbergsii* (Ramirez & Gonzalez, 1984). Classification of the genus has been confused by the use of a large number of generic synonyms e.g. *Chromotorula*, *Cryptococcus*, *Mycotorula*, *Torula* and *Torulopsis* and an even larger number of species synonyms, although for the purpose of this thesis no attempt will be made to rename any species appearing in the literature which have names other than the eight recognised by Fell *et al.* (1984).

Cells are usually spheroidal, ovoidal or elongate with some strains producing pseudohyphae or true hyphae. As imperfect yeasts, 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). Members of the genus *Rhodotorula* form characteristic red or yellow carotenoid pigments, and many give mucous colonies due to capsule production, whilst others give pasty, dry and wrinkled colonies. They are all aerobic yeasts and are unable to ferment (Fell *et al.*, 1984).

As a genus they are amongst the most widely distributed yeasts in both natural and man-made environments, and have been isolated from a diverse range of sources including soil, fresh and marine water, air, live and decaying plant tissue, insects, brine

shrimp, fish, birds, beer, preserved food, oil drillings, animal tissues and faeces (Fell *et al.*, 1984). Moreover, they are invariably found during examination of natural yeast populations e.g. *R. glutinis*, *R. graminis*, *R. minuta*, *R. mucilaginosa*, *R. rubra* and *R. texensis* have been isolated from polluted water and sewage (Cooke *et al.*, 1960) and, in a separate study of the South Saskatchewan River, *Rhodotorula* species constituted 30 out of the 129 yeasts isolated (Spencer *et al.*, 1970). Probably the main reason for their wide-spread distribution is their nutritional diversity, with many strains being able to grow on a large number of carbon substrates. For example, *R. graminis* strain KGX 39 can grow on glucose, galactose, maltose, mannitol, ribitol, citrate and succinate as well as on aromatic compounds such as benzyl alcohol, mandelate, phenylalanine, protocatechuate and salicylate (Fell *et al.*, 1984; Durham *et al.*, 1984; Middleton, 1988). Indeed, species of *Rhodotorula* have often been isolated during studies of the utilisation of aromatic compounds by yeasts. Mills *et al.* (1971) isolated yeasts from sewage and found that several strains of *R. minuta*, *R. glutinis* and *R. rubra* could grow on a variety of aromatic compounds including resorcinol, phloroglucinol, salicylate and gentisate, with one strain of *R. glutinis* also using phenol as a sole source of carbon and energy.

Studies on the utilisation of lignin, the second most abundant natural bio-polymer after cellulose, have also revealed that species of *Rhodotorula* are capable of utilising compounds associated with its degradation. Strains of *R. macerans* and *R. mucilaginosa* can grow on 4-hydroxybenzoate, ferulate and vanillate, with *R. mucilaginosa* also being able to use coumarate, all of which are breakdown products of beech, pine and spruce lignins (Cain *et al.*, 1968; Cook & Cain, 1974). A strain of *R. glutinis* has also been found to grow on sinapate, a monomer of hard-wood lignin. However, although this yeast could degrade sinapate to syringate in the absence of glucose, further metabolism of syringate to 3-*O*-methylgallate, gallate and 2,6-dimethoxy-1,4-benzoquinone occurred only in the presence of glucose as a co-metabolite (Gupta *et al.*, 1986). Co-metabolism of aromatic compounds is also a feature of other species of *Rhodotorula*; *R. glutinis*, *R. mucilaginosa* and *R. minuta* can co-metabolise the plant phenylpropanoid, rutin (Westlake & Spencer, 1966), and a species of the related genus *Rhodosporidium* co-metabolises 4-chloronitrobenzene to 4-chloro-2-hydroxyacetanilide and 4-chloroacetanilide (Corbett & Corbett, 1981). Other species of *Rhodotorula*, whilst unable to grow on certain aromatic compounds as the sole source of carbon and energy can, nevertheless, partially degrade them. For example, a strain of *R. glutinis* grew on phenol but not on monochloro or monobromophenols, although phenol-grown cells consumed oxygen in the presence of 2-, 3-, and 4-chlorophenol, 4-bromophenol, 2,4-dichlorophenol and 2,4-dibromophenol, and converted 3- and 4-chlorophenol to 4-chlorocatechol (Walker, 1973). These examples highlight the nutritional diversity of the genus *Rhodotorula* and presumably they and other yeasts, particularly species of *Candida*, *Cryptococcus*, *Debaromyces*, *Sporobolomyces* and *Trichosporon* which also degrade a variety of aromatics (Cain *et al.*, 1968; Mills *et al.*, 1971; Gaal & Neujahr, 1979),

contribute to the overall microbial breakdown and recycling of these compounds in the environment. Moreover, this is significant since many natural and man-made aromatic compounds are recalcitrant and/or toxic (Fewson, 1981, 1988*a*).

Species of *Rhodotorula* have also received particular attention as some of their enzymes have found use in biotransformation processes. The enzyme phenylalanine ammonia lyase from *R. glutinis*, *R. graminis* and *R. rubra* catalyses the deamination of L-phenylalanine to *trans*-cinnamate and ammonia, 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, when the enzyme is incubated in the presence of high concentrations of *trans*-cinnamate and ammonia at elevated pH the reaction can be reversed and, as such, the enzyme from a species of *Rhodotorula* has been exploited in a commercial process for the production of L-phenylalanine (Hamilton *et al.*, 1985). Other biotransformations in which *Rhodotorula* species have been employed include the synthesis of chiral cyclopentenones and the monitoring of asymmetric carbonyl reduction (Allenmark & Andersson, 1988; Okano *et al.*, 1988).

Phenylalanine ammonia lyase has found potential use in the treatment of phenylketonuria. This inborn error of metabolism is usually treated by low dietary intake of phenylalanine, and studies with *R. rubra* grown on a hydrolysate of milk whey protein, but entrapped within a polypyrrole matrix, have enabled production of a low phenylalanine peptide mixture suitable as a phenylketonuria foodstuff (Watanabe *et al.*, 1988). The enzyme from *R. glutinis* has also found a possible use in the direct treatment of the condition, as it can convert phenylalanine to *trans*-cinnamate within the alimentary tract and thereby reduce the blood phenylalanine levels of patients after ingestion of a normal, non-restrictive meal (Hoskins *et al.*, 1980).

Finally, 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. Unlike their counterparts in the wild, the meat of cultivated fish lacks the characteristic pinkness because of a deficiency of the pigment astaxanthin. However, feeding salmon with the yeast supplement has been successful in restoring the pink colour to the meat. Furthermore, studies have been undertaken to determine whether yeast derived pigment is an economically viable alternative to artificial colouring or pink crustacean waste which, at present, are used by salmon farmers to obtain the desired effect (Highfield, 1987; W. Hall, personal communication).

1.4 Aims and scope of this thesis

Although several fungi can utilise mandelate as a growth substrate, none of the enzymes involved in the degradation of mandelate to benzoate seem to have been purified to homogeneity, although some of the enzymes have been studied in crude extract or in partially purified form. In contrast, all of the mandelate enzymes of the bacterium *A. calcoaceticus*; D(—)- and L(+)-mandelate dehydrogenase, phenylglyoxylate decarboxylase and benzaldehyde dehydrogenase I, have been purified and characterized. The principal aim of this project was therefore to purify and characterize the NAD^+-dependent D(—)-mandelate dehydrogenase from the yeast *R. graminis* in order to answer the questions : How similar are bacterial and fungal mandelate dehydrogenases, and what are the evolutionary relationships between them ?

The first objective was to design a purification procedure for the enzyme. Purification would in turn allow various physical, chemical and kinetic properties of the enzyme to be determined e.g. native and subunit M_r , amino acid composition, amino terminal sequence and substrate and inhibitor specificities. Comparison could then be made between the *R. graminis* D(—)-mandelate dehydrogenase and the mandelate dehydrogenases of *A. calcoaceticus*, as well as with other NAD^+-dependent dehydrogenases. In addition, antisera raised against the yeast enzyme would enable an immunological comparison of the various mandelate dehydrogenases. A subsidiary aim of this project was to determine whether other yeasts were capable of growth on mandelate, and to isolate mandelate-utilising yeasts by selective enrichment from natural environments.

During the course of the present work, the aims were expanded to include experiments designed to identify amino acid residue(s) at the active site of D(—)-mandelate dehydrogenase which might be involved in catalysis. It was thought that the synthesis and purification of a bromoacetyl derivative of the substrate D(—)-mandelate would enable the active site to be probed for reactive and accessible nucleophilic groups. Furthermore, isolation of peptides derived from enzyme inactivated with a radiolabelled form of the inhibitor would allow purification and sequencing of peptide(s) corresponding to regions at or near the active site.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemicals were of the best quality commercially available and most of them were obtained from BDH Chemicals Ltd, Poole, Dorset, UK, except for those listed below.

Aldrich Chemical Company Ltd, Gillingham, Dorset, UK : anhydrous acetonitrile, anhydrous pyridine, bromoacetic acid, bromoacetylbromide, D,L-4-chloromandelic acid, D(—)-hexahydromandelic acid, isobutylchloroformate, thiopheneglyoxylic acid.

Amersham International, Little Chalfont, Bucks., UK : bromo[1-^{14}C]acetic acid, glucose[1-^{3}H].

Bethesda Research Laboratories, Gaithersburg, MD, USA : urea.

Boehringer Corporation, Lewes, Sussex, UK : DTT, NAD^+ (free acid), $NADP^+$ (disodium salt), NADH (disodium salt), NADPH (disodium salt), oxaloacetic acid, 2-oxoglutaric acid, Tris (base).

Fluka Chemicals Ltd, Glossop, Derbyshire, UK : D(—)-mandelic acid, L(+)-mandelic acid, phenylglyoxylic acid.

Formachem (Research International) Ltd, Strathaven, Midlothian, UK : $(NH_4)_2SO_4$.

FSA Laboratory Supplies, Loughborough, Leics., UK : acetic acid, acrylamide, ethanediol, formic acid, *N*,*N*′-methylene-*bis*-acrylamide, NaCl, orthophosphoric acid, SDS, sucrose.

G.T. Gurr Ltd, London, UK : Pyronin Y.

James Burrough (F.A.D.) Ltd, Witham, Essex, UK : ethanol.

Koch Light Laboratories Ltd, Colnbrook, Bucks., UK : D,L-4-fluoromandelic acid, D,L-phenyllactic acid, phenylpyruvic acid, trichloroacetic acid.

May & Baker Ltd, Dagenham, Essex, UK : ethyl acetate, nitric acid, pyridine, toluene.

Rathburn Chemicals, Walkerburn, Peebleshire, UK : dichloromethane, 4-methylmorpholine, TFA.

Riedel de-Haen AG, Seelze, Hannover, FRG : Na_2CO_3.

Scientific Marketing Association, London, UK : leupeptin.

Serva Feinbiochemica, Heidelberg, FRG : Brilliant Blue G-250.

Sigma Chemical Company, Poole, Dorset, UK : acetyl-CoA (lithium salt), ADP (disodium salt), ATP (disodium salt), bathophenanthroline disulphonic acid, benzamidine, Bicine, chloramphenicol, diethyldithiocarbamic acid, dihydroxybenzene disulphonic acid, 2,2′-dipyridyl, 5,5′-dithiobis(2-nitrobenzoic) acid, *N*-ethylmaleimide, fluorescamine, guanidine hydrochloride, D,L-2-hydroxycaproic acid, D,L-2-hydroxyisocaproic acid, D,L-3-hydroxymandelic acid, D,L-4-hydroxymandelic acid, D,L-4-hydroxy-3-methoxymandelic acid, D,L-3-hydroxy-4-methoxymandelic acid, D,L-4-hydroxyphenyllactic acid, D,L-2-hydroxyvaleric acid, D,L-2-hydroxyisovaleric acid, 8-hydroxyquinilone, D(—)-lactic acid, iodoacetamide, D(+)-malic acid, Mes, Mops, Nitro Blue Tetrazolium, 2-oxovaleric acid, 2-oxoisovaleric acid, 2-oxoisocaproic acid, 2-oxooctanoic acid, 2-oxo-3-methylvaleric acid, pepstatin, PMSF, pyruvic acid, sodium azide, Tween 20, 4-chloro 1-naphthol.

2.1.2 Chromatography media

Amicon Ltd, Stonehouse, Glos., UK : Dyematrex screening kit and Matrex Gel Orange A.

Pharmacia Ltd, Milton Keynes, Bucks., UK : DEAE-Sephacel, Phenyl Sepharose, Sephadex G-50 and pre-packed FPLC Mono P (chromatofocussing) and Superose 6 (gel filtration) columns.

Sigma Chemical Company, Poole, Dorset, UK : Blue Sepharose CL-6B.

2.1.3 Proteins and enzymes

BDH Chemicals Ltd, Poole, Dorset, UK : *Saccharomyces cerevisiae* alcohol dehydrogenase.

Boehringer Corporation, Lewes, Sussex, UK : pig heart lactate dehydrogenase, rabbit muscle pyruvate kinase, horse spleen ferritin and horse heart cytochrome c.

Fluka Chemicals Ltd, Glossop, Derbyshire, UK : *S. cerevisiae* alcohol dehydrogenase.

Pharmacia Ltd, Milton Keynes, Bucks., UK : 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.

Sigma Chemical Company, Poole, Dorset, UK : rabbit muscle aldolase, ovalbumin, *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase and *S. cerevisiae* hexokinase.

Wilfred Smith Ltd, Edgeware, London, UK : bovine serum albumin (fraction V).

Worthington Biochemical Corporation, Freehold, NJ, USA : trypsin.

2.1.4 Miscellaneous materials

British Oxygen Corporation, Guildford, Surrey, UK : N_2 , O_2.

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

Fluka Chemicals Ltd, Glossop, Derbyshire, UK : Silica Gel 60.

Gibco, Paisley, Strathclyde, UK : heat-inactivated horse serum.

Macherey-Nagel, Duren, FRG : Polygram Sil G TLC plates.

Merck, Darmstadt, FRG : Kieselgel 60 F_{254} TLC plates.

Pharmacia Ltd, Milton Keynes, Bucks., UK : Polybuffer PB 74.

Schleicher & Schuell, Dassel, FRG : nitrocellulose.

Scottish Antibody Production Unit, Carluke, Lanarkshire, UK : normal donkey serum, normal rabbit serum, donkey anti-rabbit IgG peroxidase conjugate.

The Scientific Instrument Centre, Eastleigh, Hants, UK : visking tubing.

Whatman International Ltd, Maidstone, Kent, UK : chromatography and filter papers.

2.2 General methods

2.2.1 pH measurements

The pH values of solutions were usually determined with an E.I.L. model 7010 pH monitor (E.I.L. Ltd, Cumbernauld, Strathclyde, UK) connected to a combined glass electrode (Probion Ltd, Glenrothes, Fife, UK). Small volumes, e.g. 1 ml assay mixtures, were measured with a Radiometer (Copenhagen, Denmark) type M26 pH monitor fitted with a GK 2302 micro pH electrode.

2.2.2 Conductivity measurements

The conductivities of solutions were determined with a Radiometer type CDM2e conductivity meter.

2.2.3 Glassware

Glassware was washed by immersion in a solution of Haemo-sol [Alfred Cox (Surgical) Ltd, Coulsdon, Surrey, UK] according to the manufacturer's instructions, and then thoroughly rinsed with tap water and then distilled water. Glassware for chemical syntheses was baked overnight in an oven at 110°C. Glassware for protein chemistry was immersed in boiling 10% (v/v) nitric acid for 30 min, followed by thorough rinsing with distilled water.

2.2.4 Dialysis

Dialysis tubing was prepared by boiling for 10 min in 1% (w/v) EDTA and then rinsed and boiled 3 times in distilled water for 10 min. The tubing was stored in 20% (v/v) ethanol.

2.2.5 Protein estimation

Protein was estimated using the methods of Lowry *et al.* (1951) and Bradford (1976). Standard curves of A_{750} (Lowry) or A_{595} (Bradford) against protein concentration were constructed using BSA covering the range 10 to 100 μg ml^{-1}. In both cases, unknown samples were made up to 1 ml with distilled water prior to the addition of reagents.

During enzyme purification, the A_{280} values of column effluents were measured with an LKB (Milton Keynes, Bucks., UK) Uvicord 2138 monitor. The A_{280} values of effluents from FPLC (Pharmacia) columns were measured with a Pharmacia UV-1 monitor.

2.2.6 Preparation of chromatography media

DEAE-Sephacel and Phenyl Sepharose were supplied pre-swollen and were poured directly into columns. Matrex Gel Orange A was washed with 5M-urea to remove unbound dye from the support matrix before use. Columns were equilibrated by washing with 10 volumes of the appropriate buffer. DEAE-Sephacel and Matrex Gel Orange A were regenerated after use by washing with 1M-NaCl, and Phenyl Sepharose was regenerated with 5M-urea. After the Orange A had been used several times it was fully regenerated by

successive washes in 1 M-NaCl, 5 M-urea and 50% (v/v) chloroform in methanol. All columns were stored in 0.02% sodium azide, except the Superose 6 column which was stored in 20% (v/v) ethanol.

2.2.7 Lyophilization

Samples for protein chemistry were frozen in appropriate vessels by placing them into methanol/dry ice. The tops were covered with Nescofilm (Nippon Shoji Kaisha Ltd, Osaka, Japan) punctured with a needle. Frozen samples were placed into a dessicator attached to a Flexi-dry lyophilizer (F.T.S. Systems Inc., Stone Ridge, NY, USA) and the dessicator evacuated with a Javac (Farnham, Surrey, UK) vacuum pump.

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

2.2.8 Scintillation counting and autoradiography

Radioactivity (^{14}C and ^{3}H) was measured using an LKB model 1209 Rackbeta liquid scintillation counter. Samples (maximum volume 500 μl) were added and thoroughly mixed with 5 ml or 10 ml of Ecoscint A (National Diagnostics, Manville, NJ, USA). Background radiation was measured by determining the counts from an equal volume of solvent not containing the radioactive sample.

TLC plates were autoradiographed by overlaying with Fuji RX-100 X-ray film. After exposure, the film was developed with a Kodak (Hemel Hempstead, Herts., UK) ME-3 X-OMAT processor according to the manufacturer's instructions.

2.3 Microorganisms; maintenance, growth, harvesting and disruption

2.3.1 Source of microorganisms

Yeasts

Rhodotorula graminis strain KGX 39 was obtained from Dr D. R. Durham, Genex Corporation, Gaithersburg, MD 20877, USA, and was originally isolated from soil by enrichment culture using benzoate as the sole source of carbon (Durham *et al.*, 1984).

Rhodotorula graminis (NCYC 502, 980 & 1401), *Lloderomyces elongisporus* (NCYC 928), *Rhodotorula pilimanae* (NCYC 759), *Rhodotorula minuta* (NCYC 931) and *Candida tropicalis* (NCYC 1503) were from the National Collection of Yeast Cultures, Norwich, Norfolk, UK.

Pachysolen tannophilus (NCYC 614), *Rhodotorula glutinis* (NCYC 59), *Rhodosporidium toruloides* (NCYC 819), *Candida utilis* (NCYC 321), *Candida nitratophila* (NCYC 556), *Hansenula anomala* (NCYC 432) and *Sporobolomyces roseus* (NCYC 1609) were obtained from Dr C. R. Hipkin, School of Biological Sciences, University College of Swansea, Wales.

Candida pseudotropicalis (NCPF 3234) was obtained from Mr I. Watt, Department of Microbiology, University of Glasgow.

Trigonopsis variabilis (CBS 1040), *Torulopsis colliculosa* (CBS 133), *Hanseniaspora valbyensis* (CBS 479), *Debaromyces hansenii* (CBS 767), *Wingia robertsii* (CBS 2934), *Saccharomyces bayanus* (CBS 380), *Wickerhamia fluorescens* (CBS 4565), *Trichosporon cutaneum* (CBS 2466) and *Pichia membranaefaciens* (CBS 107) were obtained from Ms E. Scott, Department of Medical Mycology, University of Glasgow.

Rhodotorula grinbergsii (IJFM 6000) was obtained from Professor C. Ramirez, Thailand Institute of Scientific and Technological Research, Bangkok 10900, Thailand.

Rhodotorula nothofagii (CCY 20-19-1) was obtained from Dr E. Slavikova, Czechoslovak Collection of Yeasts, Institute of Chemistry, Bratislava 842 38, Czechoslovakia.

Soil, compost, silage and animal faeces for enrichment of mandelate-utilising yeasts were collected with the help of Mr D. M^{c}Kellar, Ms P. M^{c}Kellar and Mr S. MacKenzie.

Bacteria

Acinetobacter calcoaceticus mutant strain C1219 [NCIB 11457, constitutive for D(—)-mandelate dehydrogenase, L(+)-mandelate dehydrogenase and phenylglyoxylate decarboxylase] was obtained from Professor C. A. Fewson, Department of Biochemistry, University of Glasgow.

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

A crude extract of *Lactobacillus curvatus* (DSM 20 019) was obtained from Dr W. Hummel, Institute for Enzyme Technology, University of Dusseldorf, FRG.

2.3.2 Maintenance of cultures

All yeasts except those obtained from the Department of Medical Mycology, University of Glasgow 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. Those from the Department of Medical Mycology were supplied as actively growing cultures on Oxoid (Oxoid Ltd, Basingstoke, Hants, UK) Sabouraud-dextrose agar, and were used within a week of being received.

A. calcoaceticus mutant strain C1219 was maintained in 10ml of sterile complex medium (MMB broth, Allison *et al.*, 1985*a*) and *S. faecalis* was maintained in 10ml of sterile Oxoid MRS broth (Man *et al.*, 1960). Both bacteria were stored at 4°C.

2.3.3 Growth media

Minimal media

(a) Mandelate salts medium : KH_2PO_4 (2 g), $(NH_4)_2SO_4$ (1 g) and D,L-mandelic acid (0.75 g or 1.5 g) were dissolved in distilled water, the pH adjusted to 7.0 with NaOH and the volume made up to 1 l. The medium was then autoclaved and, after cooling, 20 ml of sterile 2% (w/v) $MgSO_4.7H_2O$ was added.

(b) Yeast nitrogen base/mandelate medium : D,L-Mandelic acid (0.75 g or 1.5 g) and 6.7 g of Difco yeast nitrogen base (Difco Laboratories, Detroit, MI, USA) were dissolved in distilled water, the pH adjusted to 6.0 with NaOH and the volume made up to 100 ml (x10 strength). The medium was filter-sterilized and 5 ml added to 45 ml of sterile distilled water giving a final concentration of 5 mM or 10 mM-D,L-mandelate respectively. Solid medium was prepared by addition of 5 ml of the x10 strength liquid medium to 45 ml of molten Oxoid bacteriological agar N° 1 (15 g l^{-1}).

Complex media

(a) MMB broth : Oxoid nutrient broth (26 g), D,L-mandelic acid (1.5 g), L-glutamic acid.HCl (0.9 g), KH_2PO_4 (2 g), $(NH_4)_2SO_4$ (1 g) and $MgSO_4.7H_2O$ (0.4 g) were dissolved in distilled water, the pH adjusted to 7.0 with NaOH and the volume made up to 1 l. When the medium was used for large scale growth of cells (10 l), poly(propylene glycol) 2025 (0.005%) was added as an antifoam.

(b) Oxoid MRS broth : Prepared according to the manufacturer's instructions.

(c) Oxoid Sabouraud-dextrose broth and agar : Prepared according to the manufacturer's instructions.

2.3.4 Sterilization

Growth media containing yeast nitrogen base were sterilized by filtration through Millipore (Watford, Herts., UK) 0.22 μm filters. All other growth media were sterilized by autoclaving at 109°C (5 p.s.i.) for 50 min (up to 500 ml) or 60 min (500 ml to 2 l). The 10 l fermenter and 20 l reservoir were autoclaved at 120°C (15 p.s.i.) for 75 min. Glass pipettes were sterilized by baking at 160°C for 105 min and plastic pipettes were sterilized with ethylene oxide (Anprolene) (H. W. Anderson Products Ltd, Clacton-on-Sea, Essex, UK).

The efficiency of sterilization was monitored using the appropriate Browne's tube (A. Browne Ltd, Leicester, Leicestershire, UK), except for ethylene oxide sterilization where Anprolene exposure indicators were used.

2.3.5 Growth of microorganisms

Growth of yeasts

(a) Growth of yeasts in minimal medium : Yeasts maintained as lyophilized cultures were rehydrated with sterile distilled water and subcultured onto Sabouraud-dextrose agar and grown at 23°C or 30°C for 2 to 5 days depending on the species. A loopful of culture was aseptically transferred to 50 ml of minimal medium (in a 250 ml conical flask) and shaken (200 r.p.m.) at 23°C or 30°C for 24 h, 48 h, 72 h or 96 h depending on the species.

(b) Growth of *R. graminis* KGX 39 in MMB broth : When large amounts of cells were required, *R. graminis* KGX 39 was grown in MMB broth in a 10 l fermenter fitted with a 20 l reservoir. The method used was essentially that described by Allison *et al.* (1985*b*) for the growth of *A. calcoaceticus*. An inoculum was prepared by aseptically transferring a loopful of culture from the solid medium to 200 ml of the complex medium (in a 1 l conical

flask) and shaking (200 r.p.m.) at 30°C for 19 h. The entire culture was used to inoculate the fermenter (Braun Biostat V; F.T. Scientific Instruments, Tewkesbury, Glos., UK), which contained 10 l of the complex medium. The fermenter was operated at 30°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 transferred from the reservoir. After a further 24 h growth, 9.5 l of culture were removed for harvesting and the remaining fresh medium transferred to the fermenter. The culture was grown for a final 24 h whereupon all the culture was removed and harvested.

Growth of bacteria

A. calcoaceticus strain C1219 was grown in MMB broth. Stock culture (0.1 ml) was aseptically transferred to 50 ml of MMB broth (in a 250 ml conical flask) and shaken (200 r.p.m.) at 30°C overnight. Then the entire culture was aseptically transferred to 400 ml of fresh MMB broth (in a 2 l conical flask) and the culture shaken (200 r.p.m.) at 30°C overnight. The cells were then harvested.

S. faecalis was grown in MRS broth. Stock culture (0.5 ml) was aseptically transferred to 50 ml of MRS broth (in a 250 ml conical flask) and shaken (200 r.p.m.) at 30°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 shaken (200 r.p.m.) at 30°C overnight. The cells were then harvested.

2.3.6 Optical density measurements

The optical densities of cultures were determined by measuring the OD_{595}, relative to the centrifuged medium, with a Pye-Unicam (Cambridge, Cambs., UK) SP30 spectrophotometer. Samples were diluted with water so that the OD_{595} did not exceed 0.4, and the medium was diluted by the same factor.

2.3.7 Harvesting cells

All harvesting was carried out at 4°C. Cultures (up to 50 ml) were harvested by centrifugation at 12 000 ***g*** for 15 min in an M.S.E Highspeed 18 centrifuge (M.S.E. Ltd, London, UK), whereas larger volumes were harvested at 5 000 r.p.m. for 20 min in an M.S.E. Mistral 6L centrifuge. Pellets were resuspended in a volume of cold water equal to the original culture volume and recentrifuged. The pellets were combined, made to 50 ml with cold water and centrifuged at 12 000 ***g*** for 30 min in an M.S.E. Highspeed 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°C.

(a) Ultrasonic disruption of bacteria : Cell pellets were suspended in 3 volumes of the appropriate buffer and broken with a Lucas-Dawes (London, UK) ultrasonic probe, operated at 80 W for 5 x 30 s periods alternating with cooling periods of 30 s.

(b) French pressure cell disruption of yeasts : Cell pellets were suspended in 3 volumes of the appropriate buffer and broken by 3 passages through a French pressure cell (FA-073 or FA-003 models; American Instrument Company, Silver Spring, MD, USA) at a pressure of 98 MPa.

2.3.9 Enrichment and selection of mandelate-utilising yeasts

Samples for enrichment (approx. 1 g) were suspended in sterile distilled water (10 ml) and mixed. Suspensions (1 ml) were aseptically transferred to 50 ml of yeast nitrogen base containing 5 mM-D,L-mandelate and chloramphenicol (100 mg l^{-1}) to inhibit bacterial growth. Flasks were shaken (200 r.p.m.) at 23°C until growth was observed. Samples from the first passage (1 ml) were transferred to 50 ml of fresh medium and incubated under the same conditions; a third passage was carried out where growth was marked. Appropriately diluted samples of all passages (in sterile distilled water) were plated onto yeast nitrogen base/mandelate/chloramphenicol agar plates and incubated at 23°C until growth developed. Representative colonies were selected and inoculated onto fresh selective media and Sabouraud-dextrose agar and grown at 23°C.

2.4 Enzyme assays

2.4.1 Equipment and definition of enzyme units

An LKB Ultrospec spectrophotometer was routinely used during enzyme purification and a Pye-Unicam SP8-100 spectrophotometer was used for enzyme characterization and kinetic experiments. Assays were carried out in 1 ml plastic cuvettes with a 1 cm light path at 27°C (300 K). Solutions were dispensed using Gilson (Gilson Medical Electronics, Villiers-le-Bell, France) Pipetman pipettes and Finn variable pipettes [Jencons (Scientific) Ltd, Leighton Buzzard, Beds., UK]. Volumes of less than 5 μl were dispensed with a Hamilton (Hamilton Corporation, Reno, NV, USA) glass syringe. Assays were initiated by the addition of substrate and rates followed for 3 to 5 min. Blank assays were run containing all constituents except substrate, although in some cases a non-enzyme blank was also run.

One unit of enzyme activity is defined as 1 μmol of substrate converted min^{-1}; specific activities are given as units $(mg\ of\ protein)^{-1}$. The molar absorption coefficient of NADH at 340 nm is $6.3 \times 10^3\ M^{-1}\ cm^{-1}$ (Bergmeyer, 1985), and the molar absorption coefficient of DCIP at 600 nm (pH 7.5) is $21.54 \times 10^3\ M^{-1}\ cm^{-1}$ (Armstrong, 1964).

2.4.2 Assay of NAD^+-dependent D(—)-mandelate dehydrogenase

(a) For enzyme purification the oxidation of D(—)-mandelate was measured in an assay mixture containing :

660 μl of 300 mM-glycine/NaOH buffer (pH 9.5) (200 mM assay concentration)
50 μl of 10 mM-NAD^+ (500 μM 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.

For enzyme characterization, assays were developed to measure the D(—)-mandelate dehydrogenase activity (oxidation/forward reaction) and the phenylglyoxylate reductase activity (reduction/reverse reaction).

(b) The oxidation of D(—)-mandelate was measured in a mixture 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.

(c) The reduction of phenylglyoxylate was measured in a mixture containing :

800 μl of 250 mM-potassium phosphate/NaOH buffer (pH 5.85)
(200 mM assay concentration)
50 μl of 4 mM-NADH (200 μM assay concentration)
50 μl of freshly prepared 20 mM-phenylglyoxylate (pH 7.0)
(1 mM assay concentration)
Enzyme
Distilled water to 1 ml

The rate of NADH oxidation was followed at 340 nm

2.4.3 Assay of yeast dye-linked L(+)-mandelate dehydrogenase

The dye-linked L(+)-mandelate dehydrogenase activities of yeasts were assayed by measuring the reduction of DCIP at 600 nm essentially as described by Hegeman (1966*a*). The enzymes were assayed in a mixture containing :

800 μl of 82.5 mM-potassium phosphate buffer (pH 7.0)
(66 mM assay concentration)
50 μl of 1 mM-DCIP (50 μM assay concentration)
50 μl of 160 mM-L(+)-mandelate (pH 7.0) (8 mM assay concentration)
Enzyme
Distilled water to 1 ml

2.4.4 Assay of bacterial dye-linked mandelate dehydrogenase

The dye-linked D(—)- and L(+)-mandelate dehydrogenases of *A. calcoaceticus* were assayed as described by Hills and Fewson (1983a), except that the assay concentration of D(—)-mandelate used here was 4-fold greater. The reaction was followed by measuring the reduction of DCIP at 600 nm in the presence of BSA and PMS.

D(—)-Mandelate dehydrogenase was assayed in a mixture containing :

2 ml of 100 mM-potassium phosphate buffer (pH 7.5)
(66 mM assay concentration)
100 μl of 2 mM-DCIP (67 μM assay concentration)
100 μl of 10 mM-PMS (333 μM assay concentration)
200 μl of 5% (w/v) BSA
100 μl of 60 mM-D(—)-mandelate (pH 7.0) (2 mM assay concentration)
Enzyme
Distilled water to 3 ml

L(+)-Mandelate dehydrogenase was assayed exactly as for the D(—)-mandelate dehydrogenase, except that the assay contained 100 μl of 15 mM-L(+)-mandelate (500 μM assay concentration) instead of D(—)-mandelate as substrate.

2.5 Standardisation of NADH and NAD^+ concentrations

(a) The A_{340} nm of NADH solutions were determined, and the molar absorption coefficient of 6.3×10^3 M^{-1} cm^{-1} was used to convert absorbances to concentrations using the Beer-Lambert equation.

(b) The concentrations of NAD^+ solutions were determined enzymically using an alcohol dehydrogenase assay, essentially as described by Boehringer Mannheim (1973). The reduction of NAD^+ was followed at 340 nm, and the concentration of NADH at the end of the reaction was calculated as in 2.5 (a). Although the equilibrium of the reaction catalysed by alcohol dehydrogenase favours ethanol and NAD^+, under the conditions used, conversion of NAD^+ to NADH is virtually complete and the final A_{340} is a measure of the initial amount of NAD^+ added (Bergmeyer, 1985).

2.6 Standardisation of D(—)-mandelate and phenylglyoxylate

The concentrations of D(—)-mandelate and phenylglyoxylate in assay mixtures at equilibrium, were determined using a BIO-RAD (Hemel Hempstead, Herts., UK) C_4 RP-304 reverse-phase HPLC column (250 mm x 4.6 mm) pre-equilibrated in 0.1% TFA. Samples (20 μl) were injected, the compounds eluted at a flow rate of 1 ml min^{-1} and detected at 210 nm. The peaks corresponding to D(—)-mandelic and phenylglyoxylic acids were identified after spiking samples with a known amount of pure compound. Peak areas were calculated by an on-line integrator and the amount of each aromatic acid was determined from a calibration curve constructed using known amounts of compound.

2.7 Analysis of initial enzyme velocities and determination of kinetic coefficients

The K_m and V values of D(—)-mandelate dehydrogenase with respect to its substrates, were determined by the two-substrate method described by Engel (1981). The enzyme was assayed in the presence of several non-saturating concentrations of both substrates. The K_m value for each substrate, and the V value were determined when the concentration of the other substrate had been extrapolated to infinity.

The apparent K_m and V values were determined by measuring the initial velocities in the presence of several non-saturating concentrations of the first substrate, at a fixed high concentration of the second substrate. The fixed concentration of the second substrate was at least 6.5-fold more than its apparent K_m , and in most cases was over 10-fold. The kinetic data were analysed using the Direct Linear method (Eisenthal & Cornish-Bowden, 1974) using the Enzpack computer program (Williams, 1985). Although the values given in this thesis were from the Direct Linear method, the data were also analysed and compared with the values obtained using the linear plotting methods (Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee), again using the Enzpack computer program.

2.8 Standard deviation and linear regression analysis

Averages and standard deviations are given as the mean value ± 1 standard deviation about the mean, and the number of experiments used to obtain the mean and standard deviation are given in parentheses. Linear regression was done by the least mean squares method using the Apple Macintosh Cricket Graph computer program.

2.9 Enzyme purification

All steps were carried out at a temperature of 0-4°C.

2.9.1 Preparation of the crude extract

Approximately 40-50 g of thawed yeast cells were suspended in 3 volumes of buffer A [50 mM-Tris/HCl buffer (pH 8.0) containing 1 mM-DTT], mixed with a glass homogeniser, and broken by 3 passages through a French pressure cell at 98 MPa [Methods 2.3.8 (b)]. The homogenate was centrifuged at 12 000 ***g*** for 30 min, the supernatant removed and further centrifuged at 64 000 ***g*** for 2 h. The high speed supernatant was filtered through Whatman (Whatman International Ltd, Maidstone, Kent, UK) GF/A glass paper to remove insoluble lipids, and then dialysed overnight against 2 x 2 l of buffer A. The dialysed extract was used as the starting material for enzyme purification.

2.9.2 Chromatography on DEAE-Sephacel

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

2.9.3 Chromatography on Phenyl Sepharose

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

2.9.4 Chromatography on DEAE-Sephacel

The Phenyl Sepharose pool (approx. 69 ml) was applied to a DEAE-Sephacel column (3.8 cm x 1.6 cm) pre-equilibrated in buffer A. Once loaded, the column was washed with buffer A until the A_{280} of the effluent returned to the baseline. The enzyme was then eluted with buffer A containing 100 mM-NaCl. The flow rate throughout was maintained at 30 ml h^{-1}. Active fractions were pooled (approx. 15 ml) and dialysed overnight against 2 l of buffer B [50 mM-potassium phosphate buffer (pH 7.5) containing 1 mM-DTT].

2.9.5 Chromatography on Matrex Gel Orange A

The dialysed pool was applied to a Matrex Gel Orange A column (15.5 cm x 2.6 cm) pre-equilibrated in buffer B. Once loaded, the pump was stopped for 30 min, and then the column washed with 200 ml of buffer B followed by 300 ml of buffer A. The flow rate was reversed and pure D(—)-mandelate dehydrogenase was eluted with buffer A containing 0.5 mM-NADH. The flow rate throughout was maintained at 60 ml h^{-1}. Active fractions were pooled (approx. 44 ml) and the enzyme concentrated either by vacuum dialysis or by dialysis against dry Aquacide 2 powder (Calbiochem Brand Biochemicals, La Jolla, CA, USA). The pool was further concentrated to 200 μl using a Centricon-30 microconcentrator (Amicon), and stored in buffer A containing 50% (v/v) glycerol at -20°C.

2.10 Polyacrylamide-gel electrophoresis

2.10.1 SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli (1970), using the following stock solutions :

Solution A : 3.0 M-Tris/HCl (pH 8.8) containing 0.23% TEMED

Solution B : 28% (w/v) acrylamide containing 0.735% *N,N'*-methylene-*bis*-acrylamide (deionised with amberlite)

Solution C : 0.1 M-Tris/HCl (pH 6.8) containing 0.8% SDS and 0.25% TEMED

Solution D : 20% (w/v) SDS

Solution E (reservoir buffer) : 25 mM-Tris/192-mM glycine containing 0.5% SDS

(a) Resolving gel [12.5% (w/v) acrylamide]

Solution A (25 ml), solution B (89.3 ml), solution D (1 ml) and distilled water (81.2 ml) were mixed and thoroughly degassed. Ammonium persulphate (150 mg) dissolved in distilled water (1 ml) was added and the mixture swirled gently during a second degassing. The mixture was poured between glass plates, the top of the gel overlaid with a thin layer of *n*-propanol and the gel left to polymerize. After the gel had set, the *n*-propanol was poured off, and the top of the gel thoroughly washed with distilled water. The distilled water was removed and the top of the gel dried with blotting paper.

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

Solution B (17.5 ml), solution C (10 ml) and distilled water (54 ml) were mixed and thoroughly degassed. Ammonium persulphate (150 mg) dissolved in distilled water (1 ml) was added and the mixture swirled gently during a second degassing. The mixture was poured on top of the resolving gel, an 18-track Teflon comb inserted and the gel left to polymerize.

(c) Sample preparation and electrophoresis conditions

Samples were prepared using the following stock solutions :

Solution F : 500 mM-Tris/HCl (pH 8.8), containing 15% (w/v) SDS, 80% (w/v) sucrose and sufficient Pyronin Y to give an intense pink colour; the solution was heated and filtered through cotton wool prior to use.

Solution G : 2 M-DTT

Solution H (M_r marker solution) : 1 vial low M_r standards (Pharmacia), distilled water (400 μl) and solution F (100 μl); the solution was heated to 100°C for 10 min prior to use.

Typically, samples were prepared for electrophoresis by mixing distilled water (10 μl), solution F (5 μl), solution G (2.5 μl) and sample. M_r standards were prepared by mixing solution G (1 μl), solution H (10 μl) and distilled water (4.5 μl). Both samples and M_r standards were heated to 100°C for 2 min prior to loading. Gels were run at 100 mA until the dye front was approximately 2 mm from the bottom of the gel. Gels were cooled by continuously pumping an ice-slurry through a baffled chamber built into the apparatus.

2.10.2 Non-denaturing PAGE

Non-denaturing PAGE was essentially the method of Laemmli (1970), except that SDS was omitted from all solutions. Gels were prepared from the following stock solutions :

Solution A : 3.0 M-Tris/HCl (pH 8.8) containing 0.23% TEMED

Solution B : 28% (w/v) acrylamide containing 0.735% *N,N'*-methylene-*bis*-acrylamide (deionised with amberlite)

Solution C : 100 mM-Tris/HCl (pH 6.8) containing 0.25% TEMED

Solution D (reservoir buffer) : 25 mM-Tris/192-mM glycine containing 0.01% β-mercaptoethanol

(a) Resolving gel [7.5% (w/v) acrylamide]

Solution A (25 ml), solution B (53.7 ml), distilled water (117.8 ml) were mixed and thoroughly degassed. Ammonium persulphate (150 mg) dissolved in distilled water (1 ml) was added and the mixture swirled gently during a second degassing. The resolving gel was cast as in Methods 2.10.1 (a).

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

Solution B (17.5 ml), solution C (10 ml) and distilled water (54 ml) were mixed and thoroughly degassed. Ammonium persulphate (150 mg) dissolved in distilled water (1 ml) was added and the mixture swirled gently during a second degassing. The stacking gel was cast as in Methods 2.10.1 (b).

(c) Sample preparation and electrophoresis conditions

Samples were mixed with glycerol (6 μl), 0.01% Bromophenol Blue (5 μl) and made up to 60 μl with reservoir buffer. Gels were electrophoresed at 45 mA for 30 min prior to samples being loaded. Samples were electrophoresed at the same current until the dye front was approximately 2 mm from the bottom of the gel. Gels were cooled as in Methods 2.10.1 (c).

2.10.3 Staining of gels

(a) Protein staining

Protein was stained using the method of Lumsden and Coggins (1977). Gels were immersed in 0.1% Coomassie Brilliant Blue G-250 in 50% (v/v) methanol/10% (v/v) acetic acid for 45 min at 60°C. Destaining was carried out in 10% (v/v) methanol/10% (v/v) acetic acid at the same temperature.

(b) Activity staining

Gels were stained for D(—)-mandelate dehydrogenase activity essentially as described by MacKintosh and Fewson (1988*a*), except that D(—)-mandelate was used as the substrate. Prior to staining, gels were soaked in 66.7 mM-sodium pyrophosphate buffer (pH 9.0) at 4°C for 30 min with gentle swirling. Gels were stained for D(—)-mandelate dehydrogenase activity by immersing in 66.7 mM-sodium pyrophosphate buffer (pH 9.0) containing 1 mM-NAD^+, 1 mM-D(—)-mandelate, 550 μM-PMS and 55 μM-Nitro Blue Tetrazolium in the dark for 10 min at 30°C.

2.10.4 Gel scanning

Gels were scanned with an LKB 2202 Ultrascan laser densitometer. Gels were overlaid with Cling-film and the beginning of the resolving gel marked with a felt-tipped pen.

2.11 Immunological methods

2.11.1 Production of polyclonal antisera

Polyclonal antisera were raised in two New Zealand white rabbits by Dr I. D. Hamilton, Department of Biochemistry, University of Glasgow according to Home Office regulations. Pure D(—)-mandelate dehydrogenase (500 μg) was emulsified in 50% (v/v) Freund's complete adjuvant and injected subcutaneously at several shaved sites along the back and hind quarters of the rabbits. After six weeks the rabbits were injected with 250 μg of pure enzyme emulsified in 50% (v/v) Freund's incomplete adjuvant. Rabbits were bled from an ear vein two weeks later, and at two week intervals thereafter. The blood was left to clot at 4°C overnight, the serum decanted and fragments removed by centrifugation. Serum was stored at -20°C.

2.11.2 Immuno-inhibition assay

Antisera raised against *R. graminis* KGX 39 D(—)-mandelate dehydrogenase (Methods 2.11.1) and antiserum previously raised against *A. calcoaceticus* C1123 D(—)-mandelate dehydrogenase (Fewson *et al.*, 1988) were used to probe several mandelate dehydrogenases in their native forms. Crude extract (100 μl) was mixed with antiserum (100 μl) and the mixture stood on ice for 1 h. Remaining enzymic activity in the supernatant was then assayed before and after centrifugation for 2 min in an Eppendorf (Poole, Dorset, UK) 3200 micro-centrifuge.

If loss of activity ensued, the cross reaction was quantified by immuno-titration. Serial dilutions of antisera [from undiluted antisera to 1:8192 in 50 mM-Tris/HCl (pH 8.0)] were prepared. Crude extracts containing mandelate dehydrogenase were appropriately diluted with 50 mM-Tris/HCl (pH 8.0) and samples (200 μl) were mixed with antisera (200 μl) at the various dilutions. The mixtures were stood on ice for 1 h and then the remaining mandelate dehydrogenase activity assayed as above. Control experiments were carried out using rabbit normal serum instead of immune serum.

2.11.3 Immuno-blotting (Western blotting)

Immuno-blotting was carried out essentially as described by Towbin *et al.* (1979).

(a) Electroblotting

Electroblotting was carried out in a solution of 190 mM-glycine/25 mM-Tris containing 20% (v/v) methanol and 0.02% SDS, using a BIO-RAD Trans-Blot tank. SDS-polyacrylamide gels were prepared and run as in Methods 2.10.1. The proteins were transferred to nitrocellulose at 70 V (constant voltage) for 4 h.

(b) Development of blots

Blots were developed using the following buffers :

Buffer A : 20 mM-Tris/HCl (pH 7.2) containing 150 mM-NaCl

Buffer B : 20 mM-Tris/HCl (pH 7.2) containing 150 mM-NaCl and 0.5% Tween 20

Buffer C : 50 ml 10 mM-Tris/HCl buffer (pH 7.2) mixed immediately prior to use with 10 ml methanol containing 30 mg 4-chloro 1-naphthol and 150 μl 4%(W/v) H_2O_2.

Blots were soaked overnight in buffer A containing 5% (v/v) normal donkey serum at 4°C, then rinsed twice with buffer A. They were then incubated in buffer B containing 5% (v/v) normal donkey serum and 0.5% antiserum for 90 min at 23°C with gentle swirling. After washing for four 12 min periods in buffer B and five 12 min periods in buffer A the blots were incubated in buffer A containing 5% (v/v) normal donkey serum and 0.05% donkey anti-rabbit IgG conjugated to peroxidase, for 90 min at 23°C with gentle swirling. Blots were rinsed twice with 10 mM-Tris/HCl (pH 7.2) and then incubated (approx. 5 min) in freshly prepared buffer C for bands to develop. They were then washed with distilled water and stored dry between sheets of filter paper.

2.12 Synthesis, purification and analysis of D(—)-2-(bromoacetyl)mandelic acid

2.12.1 Synthesis of D(—)-2-(bromoacetyl)mandelic acid

Two methods for the synthesis of D(—)-2-(bromoacetyl)mandelic acid were used. The method using bromoacetylbromide was essentially as described for the synthesis of 3-(bromoacetyl)chloramphenicol (Kleanthous *et al.*, 1985), except that D(—)-mandelic acid replaced chloramphenicol. As radiolabelled bromoacetylbromide was not commercially available, a method using bromo[1-^{14}C]acetic acid was developed.

(a) Method 1 - Synthesis using bromoacetylbromide

D(—)-Mandelic acid (1 g, 6.5 mmol) was dried under vacuum over phosphorous pentoxide for 24 h at 30°C. It was then dissolved in anhydrous acetonitrile (10 ml) at 0°C and stirred under nitrogen. Bromoacetylbromide (578 μl, 6.5 mmol) was added, followed by dropwise addition of anhydrous pyridine (531 μl, 6.5 mmol) and the mixture stirred for 30 min at 0°C. It was then stirred for a further 30 min at room temperature. Solvent was removed at 30°C by rotary evaporation under vacuum, and the oily residue remaining washed several times with acetonitrile. It was then redissolved in acetonitrile (approx. 2 ml) for purification. The reaction was monitored by TLC on silica gel plates, eluting with toluene/ethyl acetate/formic acid at 5:1:1 (v/v) and the products were visualised by their absorbance under ultra violet light.

(b) Method 2 - Synthesis using bromoacetic acid

D(—)-Mandelic acid (38 mg, 250 μmol) was dried under vacuum over phosphorous pentoxide for 24 h at 30°C. It was then dissolved in dichloromethane (50 ml) and cooled to -20°C in a dry ice/methanol bath. Bromo[1-^{14}C]acetic acid (5 μmol at 56 μCi μmol^{-1}) and bromoacetic acid (34 mg, 245 μmol) were dissolved in separate volumes of dichloromethane (2 ml and 10 ml respectively), then transferred to the reaction vessel and cooled to -20°C. 4-Methylmorpholine (27.8 μl, 250 μmol) was added, followed by

isobutylchloroformate (32.8 μl, 250 μmol), and the mixture stirred for 2 min. Then, the D(—)-mandelic acid dissolved in dichloromethane was added and the complete mixture stirred under nitrogen at -20°C for 4 h. It was finally left at -20°C overnight without further stirring. The reaction was monitored by TLC, the solvent removed and the products prepared for purification as in Methods 2.12.1 (a).

2.12.2 Purification of D(—)-2-(bromoacetyl)mandelic acid

D(—)-2-(Bromoacetyl)mandelic acid was separated from pyridine and unreacted mandelic acid using pressurised "flash" chromatography as described by Still *et al.* (1978). A column (23.5 cm x 2.5 cm) of Silica Gel 60 was packed and equilibrated with toluene at a pressure of 35 kPa (5 p.s.i.) of nitrogen. The column was then washed with 300 ml of toluene/ethyl acetate 5:1 (v/v) containing 8.3% (v/v) formic acid. Sample was applied and washed into the top of the column under gravity, the column reservoir filled with solvent and the pressure re-applied. Fractions (5 ml) were collected manually and those containing D(—)-2-(bromoacetyl)mandelic acid identified by TLC as in Methods 2.12.1 (a).

When D(—)-2-(bromo[^{14}C]acetyl)mandelic acid was purified, column fractions were also counted as in Methods 2.2.8. Solvent from the pooled fractions was removed by rotary evaporation and the D(—)-2-(bromoacetyl)mandelic acid washed several times with acetonitrile. It was finally dried under vacuum, over solid NaOH for 48 h and then stored under nitrogen at 4°C.

2.12.3 Analysis of D(—)-2-(bromoacetyl)mandelic acid

(a) Nuclear magnetic resonance spectroscopy

The ^{1}H-NMR spectrum of D(—)-2-(bromoacetyl)mandelic acid was obtained using a Bruker (Bruker Spectrospin Ltd, Coventry, West Midlands, UK) model WP 220 SY (200 MHz) NMR spectrometer, by Mr J. Gall, Department of Chemistry, University of Glasgow. The compound was dissolved in deuterochloroform and the resonances referenced to chloroform at 7.25 ppm and benzene [25% (v/v) in deuterochloroform] at 7.15 ppm.

(b) Mass spectrometry

The mass spectrum of D(—)-2-(bromoacetyl)mandelic acid was obtained using electron impact mass spectrometry with a Kratos MS-30 mass spectrometer by Dr T. Bugg and colleagues, Department of Organic Chemistry, University of Cambridge.

(c) Elemental analysis

The elemental composition of D(—)-2-(bromoacetyl)mandelic acid was determined by Ms K. Wilson, Department of Chemistry, University of Glasgow. Carbon and hydrogen were analysed using a Carlo Erba (Carlo Erba Instruments, Crawley, Sussex, UK) Model 1106 elemental analyser, whereas bromine was titrated with mercuric nitrate using bromobenzoic acid as a standard.

(d) Circular dichroism

Circular dichroism was carried out by Ms S. Kelly using a JASCO J-600 Spectropolarimeter at the Scottish CD Facility, School of Biological Sciences, University of Stirling. D(—)-2-(Bromoacetyl)mandelic acid (0.2 mg ml^{-1}, 0.74 mM) and D(—)- and L(+)-mandelic acids (0.2 mg ml^{-1}, 1.3 mM) dissolved in acetonitrile (HPLC grade), were scanned from 190-250 nm in a 0.02 cm pathlength cuvette.

2.13 Affinity labelling of D(—)-mandelate dehydrogenase with D(—)-2-(bromoacetyl)mandelate

2.13.1 Kinetics of inactivation

The kinetics of inactivation of D(—)-mandelate dehydrogenase by D(—)-2-(bromoacetyl)mandelate were determined in the absence and presence of the substrate, D(—)-mandelate. A stock solution (0.8 M) of the affinity label in acetonitrile was used to prepare the various dilutions used for inactivation.

(a) Inactivation in the absence of D(—)-mandelate

The inactivation mix (100 μl) contained D(—)-mandelate dehydrogenase (19 μg, 5 μM), D(—)-2-(bromoacetyl)mandelate (1-4 mM) and acetonitrile (0.95 M). Immediately after addition of the label, the inactivation mix was vortexed and a sample (10 μl) removed and diluted into 990 μl of 50 mM-Tris/HCl (pH 8.0). Samples (10-40 μl) were then assayed for residual phenylglyoxylate reductase activity as in Methods 2.4.2 (c). At 15 min intervals throughout the 2 h time course, samples (10 μl) were removed, diluted and assayed for residual enzymic activity. The activity of the sample taken immediately after addition of the label was taken as the 100% value. Control incubations containing enzyme, acetonitrile (0.95 M) but no inhibitor were carried out in parallel.

(b) Inactivation in the presence of D(—)-mandelate

The inactivation mix (100 μl) contained D(—)-mandelate dehydrogenase (19 μg, 5 μM), D(—)-2-(bromoacetyl)mandelate (1-8 mM), D(—)-mandelate (5 mM) and acetonitrile (0.95 M). The inactivation was carried out as in Methods 2.13.1 (a).

2.13.2 Stoichelometry of inactivation

A stock solution (63 mM) of D(—)-2-(bromo[^{14}C]acetyl)mandelic acid in acetonitrile, with a specific activity of 1.12 nCi $nmol^{-1}$ was used for this experiment and those given in Methods 2.13.3. The inactivation mix (1125 μl) contained D(—)-mandelate dehydrogenase (1.3 mg, 30.4 μM), D(—)-2-(bromo[^{14}C]acetyl)mandelate (1.4 mM) and acetonitrile (420 mM). Immediately after addition of the label, the inactivation mix was vortexed and a sample (5 μl) removed and diluted into 995 μl of 50 mM-Tris/HCl (pH 8.0). This was further diluted (250 μl into 750 μl of the same buffer) before samples (10 μl) were assayed for phenylglyoxylate reductase activity as in Methods 2.4.2 (c). At intervals throughout the 3 h inactivation, 5 μl were removed, diluted and appropriate samples (10-100 μl) assayed for residual activity. The activity of the sample taken immediately after

addition of the label was taken as the 100% value. Covalent incorporation of label was monitored by a filter disc method and gel filtration; removing samples from the inactivation mix at the same time as those removed for assay of residual activity.

Filter disc method : Samples (50 μl) were applied to 2.5 cm diameter circles of Whatman 3MM filter paper and the protein precipitated by immersion in 5% (w/v) ice-cold trichloroacetic acid. Unbound counts were removed by washing the discs with fresh ice-cold trichloroacetic acid for 5 min, then with ice-cold ethanol : diethyl ether 1:1 (v/v) for 10 min and finally with diethyl ether at room temperature for 10 min before being left to dry. The filter discs were counted (Methods 2.2.8) and corrections made for quenching by the paper and the protein applied to it. The amount of protein applied to each disc was calculated from the initial protein concentration prior to addition of the affinity label.

Gel filtration : Samples (100 μl) were gel filtered through a column (30 cm x 1.5 cm) of Sephadex G-50 at a flow rate of 99 ml h^{-1}. Fractions (3 ml) were collected, and the location and concentration of the protein determined by the Bradford method (Methods 2.2.5). The bound label was measured by counting samples (500 μl) of the fractions containing protein (Methods 2.2.8).

2.13.3 Identification and preparation of labelled peptides

In order to identify the labelled active site peptides, D(—)-mandelate dehydrogenase was inactivated by D(—)-2-(bromo[^{14}C]acetyl)mandelate in the absence and presence of the substrate, D(—)-mandelate.

(a) Inactivation in the absence of D(—)-mandelate

The inactivation mix (371 μl) contained D(—)-mandelate dehydrogenase (500 μg, 35.6 μM), D(—)-2-(bromo[^{14}C]acetyl)mandelate (230 μM) and acetonitrile (69 mM). Immediately after addition of the label the inactivation mix was vortexed and a sample (5 μl) removed, diluted and assayed for enzymic activity as in Methods 2.13.2. Residual enzymic activity was monitored throughout the 4 h inactivation.

(b) Inactivation in the presence of D(—)-mandelate

The inactivation mix (462 μl) contained D(—)-mandelate dehydrogenase (500 μg, 28.6 μM), D(—)-2-(bromo[^{14}C]acetyl)mandelate (232 μM), D(—)-mandelate (100 mM) and acetonitrile (70 mM). The inactivation was carried out as in Methods 2.13.3 (a).

(c) Inactivation of D(—)-mandelate dehydrogenase for peptide purification

The inactivation mix (1.3 ml) contained D(—)-mandelate dehydrogenase (5 mg, 100 μM), D(—)-2-(bromo[^{14}C]acetyl)mandelate (2.4 mM) and acetonitrile (0.73 M). Samples (5 μl) were removed, diluted and the residual enzymic activity measured as in Methods 2.13.2.

(d) Preparation of inactivated protein for trypsin digestion

Covalently labelled enzyme was separated from unbound label by gel filtration through a column (30 cm x 1.5 cm) of Sephadex G-50 at a flow rate of 99 ml h^{-1}. Fractions (3 ml) were collected and the radioactivity and A_{280} of each fraction measured. Fractions containing protein were pooled, frozen and lyophilized. To ensure cleavage of the ester

link between mandelate and the carboxymethylated protein, the protein was dissolved in 0.1M-Tris/HCl (pH 8.5) containing 6M-guanidine hydrochloride (1 ml) and 28 mM-β-mercaptoethanol, and stirred for 2 h at 37°C. Then, 1M-NaOH (110 μl) was added and the mixture stirred for a further 6h at 37°C. The protein was separated by gel filtration, the radioactivity and A_{280} of the fractions measured, and those containing protein were pooled, frozen and lyophilized.

2.14 Synthesis of tritiated NADH

Experiments involving the synthesis and use of tritiated NADH were carried out in collaboration with Mr E. Weinhold, Laboratory for Organic Chemistry, ETH-Zentrum, CH-8092, Zurich, Switzerland.

(a) Synthesis of tritiated pro-*S* NADH

This method relies on the fact that *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase specifically transfers a hydride ion (H^- or $^3H^-$ in the case of tritium) from glucose 6-phosphate to the pro-*S* position of NAD^+ or $NADP^+$ (Arnold *et al.*, 1976).

The tritiated glucose 6-phosphate was produced by phosphorylation of glucose[1-^{3}H] by ATP using *S. cerevisiae* hexokinase. The linked assay was carried out in a mixture (500 μl) containing : 5mM-potassium phosphate buffer (pH 8.0), 60.5 μM-glucose[1-^{3}H] (specific activity 1.9 Ci mmol^{-1}), 500 μM-ATP, 3.7 units of hexokinase, 60.5 μM-NAD^+ and 55 units of glucose-6-phosphate dehydrogenase. The rate of NAD^+ reduction was followed at 340 nm and the reaction was assumed to be at equilibrium when the progress curve reached a plateau. Protein was then removed from the mixture by ultra-filtration using a Centricon-10 microconcentrator (Amicon). The tritiated pro-*S* NADH was used immediately to reduce the incorporation of label at the pro-*R* position, caused by random exchange with the solvent.

(b) Synthesis of tritiated pro-*R* NADH

This method relies on the fact that *S. cerevisiae* alcohol dehydrogenase specifically removes the pro-*R* hydrogen from NADH (Fisher *et al.*, 1953; Vennesland, 1956). Tritiated NAD^+ was produced by incubating pro-*S* tritiated NADH with alcohol dehydrogenase and acetaldehyde. The tritiated NAD^+ was then incubated with non-radiolabelled glucose 6-phosphate and glucose-6-phosphate dehydrogenase, which transfers a hydride ion to the pro-*S* position thereby displacing the tritium to the pro-*R* position.

To a filtered reaction mixture containing pro-*S* tritiated NADH [Methods 2.14 (a)] was added 1 M-acetaldehyde (10 μl) and alcohol dehydrogenase (10 units). The rate of NADH oxidation was followed at 340 nm and the reaction was assumed to be at equilibrium when the curve reached a plateau. Protein was then removed from the mixture using a Centricon-10 microconcentrator. The tritiated NAD^+ was then purified by HPLC using an LKB DEAE-5PW Glas-Pac column (75 mm x 8 mm) run isoc ratically with 10 mM-formic acid to elute NAD^+, followed by a linear 0-500 mM-NaCl gradient to elute ADP and ATP. Fractions containing the NAD^+ were collected and lyophilized.

Tritiated pro-*R* NADH was then produced in a mixture (500 μl) containing : 10 mM-potassium phosphate (pH 8.0), 8.4 μM ^{3}H-NAD^{+}, 50 μM-glucose 6-phosphate and 28 units of glucose-6-phosphate dehydrogenase. The reaction was followed at 340 nm and the protein removed as in Methods 2.14 (a). The tritiated pro-*R* NADH was used immediately to reduce the incorporation of label at the pro-*S* position, caused by random exchange with the solvent.

(c) Incubation of tritiated NADH with D(—)-mandelate dehydrogenase

To a reaction mixture (1 ml) containing 10 mM-potassium phosphate buffer (pH 7.0), 100 μM-NADH and 100 μM-phenylglyoxylate was added either tritiated pro-*S* NADH (1.2 nmol, 2.3 μCi) or tritiated pro-*R* NADH (0.84 nmol, 1.6 μCi). The reaction was initiated by the addition of excess D(—)-mandelate dehydrogenase (3.5 μg, 92 pmol), and once the reaction was at equilibrium the protein was removed using a Centricon-10 microconcentrator. The NAD^{+} and D(—)-mandelate were separated by HPLC. Samples (500 μl) were injected into a Brownlee Laboratories (Brownlee Laboratories, Santa Clara, CA, USA) C_8 RP-300 reverse-phase HPLC column (220 mm x 4.6 mm) pre-equilibrated in 0.1% TFA. The compounds were eluted at 1 ml min^{-1} and the effluent was monitored at 220 nm and 260 nm. Fractions were collected and counted for incorporation of ^{3}H as in Methods 2.2.8.

2.15 Protein chemistry

2.15.1 Trypsin digestion

Protein was suspended in 3 ml of 50 mM-ammonium bicarbonate (pH 8.5), and trypsin (10 mg ml^{-1} in 0.1 mM-HCl) was added at a ratio of protease : enzyme of 1: 50 (w/w). The digest was stirred for 4-5 h at 37°C, after which the peptides were frozen and lyophilized.

2.15.2 Separation of peptides by HPLC

Peptides were separated by HPLC using a Waters (Watford, Herts., UK) μBONDAPAK C_{18} reverse-phase column. Peptides were dissolved in the appropriate solvent and then centrifuged to remove insoluble material. They were then injected (100 μl) onto the column and separated using one of three gradient systems, unless otherwise stated.

System I : 90% A (0.1% TFA in water) to 50% B (0.1% TFA in acetonitrile) in 60 min.

System II : 90% A [1 mM-sodium phosphate (pH 7.4)] to 40% B (acetonitrile) in 20 min.

System III : 80% A [0.05% ammoniun acetate (pH 6.0)] to 50% B (acetonitrile) in 30 min.

The flow rate throughout was 1 ml min^{-1} and the peptides were detected at 220 nm.

2.15.3 Protein amino acid composition

(a) Dialysis

Approximately 1.3 mg of purified D(—)-mandelate dehydrogenase was used to determine the amino acid composition. The enzyme was dialysed at 4°C for seven 24 h periods against 2 l of 5 mM-potassium phosphate buffer (pH 7.5), then dialysed for two 5 h periods against 2 l of distilled water.

(b) Hydrolysis

Samples (approx. 130 μg, 3.4 nmol) of dialysed enzyme were lyophilized and then dissolved in 1 ml of 5.65 M-HCl. Tubes were flushed with nitrogen, frozen and sealed under vacuum. Samples were hydrolysed at 110°C for 24 h, 48 h, 72 h and 96 h.

(c) Performic acid oxidation

Cysteine was detected as cysteic acid after oxidation with performic acid. Performic acid was prepared by mixing 30% (w/v) H_2O_2 (1 ml) and formic acid (9 ml) and left for 60 min at room temperature followed by 15 min at 0°C prior to use. Lyophilized enzyme (approx. 130 μg, 3.4 nmol) was dissolved in performic acid (1 ml) and oxidised for 4 h at room temperature. Samples were then lyophilized and washed and dried 3 times with distilled water (500 μl). The oxidised enzyme was hydrolysed for 24 h as in Methods 2.15.3 (b).

(d) Analysis

After hydrolysis, all samples were lyophilized and then washed and dried 3 times with distilled water (500 μl) over solid NaOH. D(—)-Mandelate dehydrogenase hydrolysates were dissolved in 125 μl of 0.2 N-sodium citrate buffer (pH 2.2) and samples (25 μl) analysed with an LKB 4400 amino acid analyser, by Mr J. Jardine, Department of Biochemistry, University of Glasgow. Valine, leucine and isoleucine were determined from their respective amounts at the end of the time course; serine and threonine were determined by extrapolating back to zero time. Glutamine and asparagine were determined as glutamate and aspartate respectively. The data were normalized to alanine = 23 nmol.

2.15.4 Peptide amino acid composition

Peptide amino acid analysis was carried out by Dr D. Campbell, Department of Biochemistry, University of Dundee, and by Dr J. Keen, SERC Sequencing Unit, Department of Biochemistry, University of Leeds both using a Waters PICO-TAG amino acid analyser. Lyophilized peptide was suspended in 6 M-HCl (200 μl) containing 1% (w/v) phenol, evacuated, sealed and incubated for 24 h at 110°C. After hydrolysis, the HCl was removed under vacuum and the amino acids derivatized with phenylisothiocyanate. The PTC amino acids were then separated by reverse-phase (C_{18}) HPLC using a gradient of acetonitrile in 139 mM-acetic acid containing 3.6 mM-triethylamine (pH 6.5), using the Waters PICO-TAG method.

2.15.5 Thin-layer electrophoresis of amino acids

Thin-layer electrophoresis was carried out essentially as described by Kleanthous *et al.* (1985). Amino acids were spotted onto 20 x 20 cm TLC plates (Polygram Sil G silica) along with neutral, basic and carboxymethylated amino acid standards. The plates were electrophoresed in a Shandon (Runcorn, Cheshire, UK) flat-bed electrophoresis tank with a 10% (v/v) pyridine/0.5% acetic acid buffer (pH 6.5), at 15 mA for 3 h. Plates were then

dried, washed once with acetone, twice with acetone containing 1% (v/v) triethylamine and then stained with acetone containing 0.01% fluorescamine. The amino acid spots were visualised by their fluorescence under ultra violet light.

2.15.6 Protein sequencing

(a) Dialysis

Purified D(—)-mandelate dehydrogenase (250 μg, 6.5 nmol) was dialysed at 4°C for six 24 h periods against 2 l of 0.5% ammonium bicarbonate and then lyophilized. It was then dialysed for 24 h against 5 l of 0.1% SDS, followed by dialysis for two 24 h periods against 5 l of 0.05% SDS and then lyophilized.

(b) Sequencing

The amino terminal of D(—)-mandelate dehydrogenase was sequenced by Dr J. Keen at the SERC Sequencing Unit, Department of Biochemistry, University of Leeds, as described by Findlay *et al.* (1989).

Enzyme was dissolved in 200 mM-sodium phosphate containing 0.25% SDS, and coupled to p-phenylenediisothiocyanate glass. The glass-coupled enzyme was then sequenced by automated solid-phase Edman degradation (Laursen, 1971). The ATZ amino acids produced were converted to their respective PTH derivatives, which were identified by reverse phase (C_{18}) HPLC using a gradient of acetonitrile in 35 mM-sodium acetate buffer (pH 4.9). The PTH-amino acids were quantified at 269 nm, serine and threonine residues were confirmed by detection of their dehydro-derivatives at 313 nm.

2.15.7 Peptide sequencing

Peptides were sequenced by Dr D. Campbell, Department of Biochemistry, University of Dundee, using an Applied Biosystems (Applied Biosystems Ltd, Warrington, Cheshire, UK) 470 A/120 A gas-phase sequencer equipped with on-line PTH-analysis. Peptides were also sequenced by Dr J. Keen at the SERC Sequencing Unit, Department of Biochemistry, University of Leeds, using an Applied Biosystems 477-A liquid-pulse sequencer equipped with on-line PTH-analysis and by solid-phase sequencing as described in Methods 2.15.6 (b).

2.16 Safety

(a) Microbiological

Good microbiological practice was applied throughout the course of this study. All cultures were killed by autoclaving before disposal and spilled live cultures were immediately swabbed with 25% (v/v) *n*-propanol.

(b) Chemical

All chemicals were handled with care, especially compounds with known toxic, irritant or carcinogenic properties. Dusty compounds and volatile solvents were used in a fume cupboard.

(c) Radioactivity

The use and disposal of radioactive compounds was in compliance with the rules and guidelines laid out in the Health Physics Notes of the Radiation Protection Service, University of Glasgow.

CHAPTER 3

PURIFICATION OF D(—)-MANDELATE DEHYDROGENASE FROM *RHODOTORULA GRAMINIS* STRAIN KGX 39

3.1 Introduction

Initial studies on the metabolism of mandelate by the yeast *Rhodotorula graminis* strain KGX 39 demonstrated the presence of a soluble, NAD^+-dependent D(—)-mandelate dehydrogenase (Durham, 1984). Although some preliminary data obtained using crude extract were available, little was known about the enzyme as it had not been purified. Therefore, a method for purifying the enzyme was required, if it was to be characterized and a comparison of its properties made with other mandelate dehydrogenases and NAD^+-dependent dehydrogenases.

Before any purification was attempted, experiments were carried out to optimise the yield of cells in liquid culture and to determine the effect of various growth media on the specific activity of the enzyme in crude extracts. *R. graminis* grown in mandelate salts medium [Methods 2.3.3 (a)] had a high D(—)-mandelate dehydrogenase specific activity [e.g. 0.37 units (mg protein)$^{-1}$] but the growth yield was relatively poor. Excellent growth was observed when cells were grown in the carbohydrate rich, Sabouraud-dextrose broth. However, little or no enzymic activity could be detected even when the medium was supplemented with 10 mM-D,L-mandelate, indicating that synthesis of D(—)-mandelate dehydrogenase is subject to catabolite repression. This is supported by the observation that *R. graminis* grown in a medium containing 2 mM-glucose and 10 mM-D,L-mandelate show diauxic growth; preferentially using the glucose and then, after a 1.5 - 2 h lag period, using the mandelate as growth substrate (Durham,1984).

A complex medium giving high growth yield but which did not suppress enzyme induction was therefore sought, and experiments using MMB broth showed that it met both these criteria. Growth was substantial (350 g - 500 g wet wt. from 30 l culture) and the specific activity, although approximately half of that from cells grown in mandelate salts medium, was sufficient to finally yield mg quantities of pure enzyme.

Centrifugation experiments showed that D(—)-mandelate dehydrogenase was a soluble enzyme. When crude extract was centrifuged at 64 000 ***g*** for 2 h, typically >95% of the total activity remained in the supernatant. The procedure pelleted approximately half of the protein (2-fold purification) and all crude extracts were therefore centrifuged as such prior to further purification.

3.2 Ammonium sulphate precipitation

Ammonium sulphate precipitation was tested as a possible first step in the purification. D(—)-Mandelate dehydrogenase precipitated over a relatively broad range (50-70% saturation) with a 50% loss in activity and only a 2-fold purification. In later experiments where the ammonium sulphate was added as a saturated solution at the same pH of the buffer, the recovery of activity increased to 80% although the enzyme could not be purified more than 2-fold. The use of this technique was not included in any further purification.

3.3 Chromatography on DEAE-Sephacel (anionic exchange)

Anionic exchange chromatography was chosen as the first step in the purification. In a preliminary experiment where D(—)-mandelate dehydrogenase was eluted from DEAE-Sephacel with a linear 0-1 M-NaCl gradient, the enzyme eluted between 50 mM and 70 mM-NaCl i.e. well before the main peak of protein. A shallower gradient, 0-150 mM-NaCl, was therefore used and this gave good recovery of yield and significant purification. The conductivity of crude extract was approximately equal to that of the NaCl concentration which eluted the enzyme from the column, and it therefore proved necessary to dialyse the crude extract to ensure binding to the gel bed. Figure 3.1 shows a typical elution profile of D(—)-mandelate dehydrogenase from a DEAE-Sephacel column.

3.4 Chromatography on Phenyl Sepharose (hydrophobic interaction)

Phenyl Sepharose was chosen as the second step in the purification. In a preliminary experiment where the DEAE-Sephacel pool was loaded onto a Phenyl Sepharose column, D(—)-mandelate dehydrogenase bound and remained bound after the NaCl, in which it had been loaded, was washed out. In contrast, the majority of contaminating proteins failed to bind and it was therefore a particularly useful step.

The enzyme was then eluted by including ethanediol in the buffer, a reagent that lowers the polarity of the eluent. The enzyme eluted strongly at 40% (v/v) ethanediol, but leached slowly off the column at lower concentrations. Washing the column with buffer A containing 25% (v/v) ethanediol did result in some leaching of enzyme, but it accounted for only a few percent of the total activity loaded. Figure 3.2 shows a typical elution profile of D(—)-mandelate dehydrogenase from a Phenyl Sepharose column.

3.5 Chromatography on DEAE-Sephacel (anionic exchange)

The second DEAE-Sephacel column usually resulted in some loss of enzyme yield with little purification, but it was a rapid means of removing the ethanediol from the Phenyl Sepharose pool, and obviated the need for extensive dialysis. The step was also useful in concentrating the pool to a volume easily dialysed into Buffer B, prior to loading onto the Orange A column.

3.6 Chromatography on Matrex Gel Orange A (dye-affinity)

D(—)-Mandelate dehydrogenase was finally purified using a Matrex Gel Orange A column. In preliminary studies the enzyme also bound [in 50 mM-Tris/HCl (pH 8.0) containing 1 mM-DTT] to Matrex Gels Blue A, Green A, Red A and Blue Sepharose CL-6B (Sigma), but not to Matrex Gel Blue B. However, attempts to produce homogeneous enzyme using Blue Sepharose CL-6B and/or Matrex gel Red A, eluting with various NAD^+, NADH or NaCl concentrations proved unsuccessful.

The effect of buffer pH on the binding of the enzyme to Orange A was investigated. Samples (previously dialysed into the appropriate buffer) were loaded onto Orange A columns and washed with either 50 mM-potassium phosphate buffer (pH 5.0, 6.0 or 7.0) containing 1mM-DTT, or 50 mM-Tris/HCl (pH 8.0 or 9.0) containing 1 mM-DTT. The columns were then washed with the corresponding buffer containing 0.5 mM-NADH. At pH 9.0 the enzyme failed to bind to the column, but at pH 8.0 and below the enzyme remained bound. Washing the columns with NADH eluted the enzyme at pH 7.0 and 8.0 but not at pH 5.0 or 6.0. Therefore, pH had a marked effect on the binding of the enzyme to Orange A and also on the ability of 0.5 mM-NADH to elute it from the column. The pH dependence of binding to Orange A was exploited in the purification. Changing the buffer pH from 7.5 (buffer B) to pH 8.0 (buffer A) was essential in removing contaminant proteins from the column prior to elution with 0.5 mM-NADH. However, washing the column at the higher pH did produce some leaching of enzyme, but this never represented more than a few percent of the total activity loaded. A typical elution profile of D(—)-mandelate dehydrogenase from an Orange A column is shown in Figure 3.3.

3.7 Overall purification

D(—)-Mandelate dehydrogenase from *R. graminis* KGX 39 has been purified to homogeneity 18 times with very similar results. A typical purification is summarised in Table 3.1. The enzyme was purified in good yield, 1-2 mg (after concentration) from 40-50 g wet wt. of cells and the recovery was of the order of 50-60% of the original total activity. The enzyme was also twice successfully purified on a larger scale, yielding 9.0 mg (64% yield) and 10.4 mg (83% yield) of protein from 155 g and 186 g wet wt. of cells respectively. In these cases the size of the first DEAE-Sephacel column was increased to 9.5 cm x 5.0 cm and the total volume of the 0-150 mM-NaCl gradient increased from 600 ml to 2 l. The Phenyl Sepharose column was increased to 38.0 cm x 2.6 cm. Both the second DEAE-Sephacel and Matrex Gel Orange A columns remained the same size as in Methods 2.9.4 and 2.9.5 respectively.

3.8 Stability of purified enzyme

Purified D(—)-mandelate dehydrogenase lost negligible activity even after storage for several months at -20°C.

3.9 Purity of D(—)-mandelate dehydrogenase

Purified D(—)-mandelate dehydrogenase gave a single band of protein when it was electrophoresed under denaturing and non-denaturing conditions. The purity of extracts during enzyme purification was monitored by SDS-PAGE, and a typical purification gel is shown in Figure 3.4. Densitometer scans of purified enzyme and M_r markers are shown in Figure 3.5. When purified enzyme was electrophoresed under non-denaturing conditions

and stained for protein, a single band was observed. This co-resolved with a single band when either crude extract or purified enzyme were stained for D(—)-mandelate dehydrogenase activity (Figure 3.6).

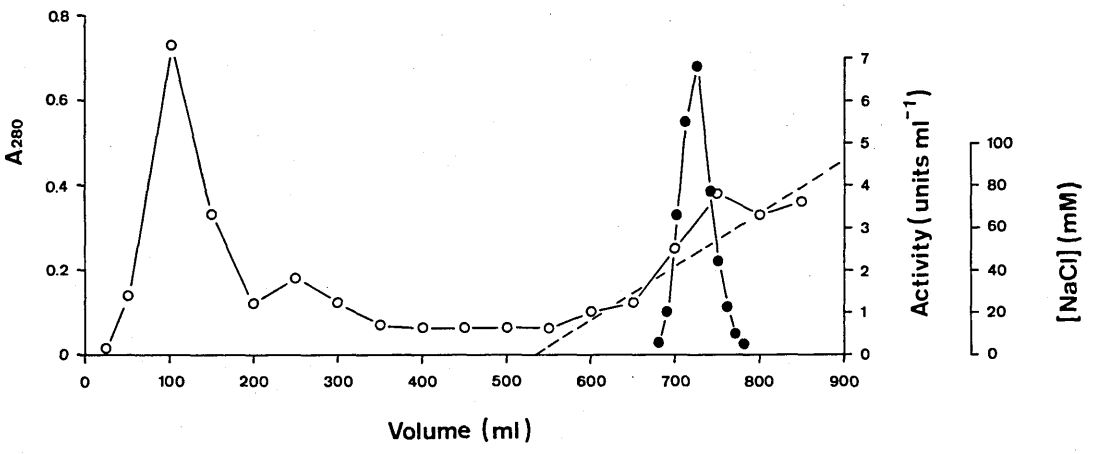


Figure 3.1 Chromatography on DEAE-Sephacel

Crude extract, approx. 97 ml (prepared as in Methods 2.9.1) was applied at 30 ml h^{-1} to a DEAE-Sephacel column (10.2 cm x 2.6 cm) pre-equilibrated in buffer A [50 mM-Tris/HCl (pH 8.0) containing 1 mM-DTT]. Once loaded, the column was washed with buffer A at 50 ml h^{-1} until the A_{280} of the effluent decreased to approximately 0.06, and then D(—)-mandelate dehydrogenase was eluted with a linear 0-150 mM-NaCl gradient (- - -) in buffer A (total volume = 600 ml) at a flow rate of 30 ml h^{-1}. The A_{280} (○) of the effluent was measured (Methods 2.2.5) and fractions (10 ml) were collected, and enzyme activity (●) assayed as in Methods 2.4.2 (a).

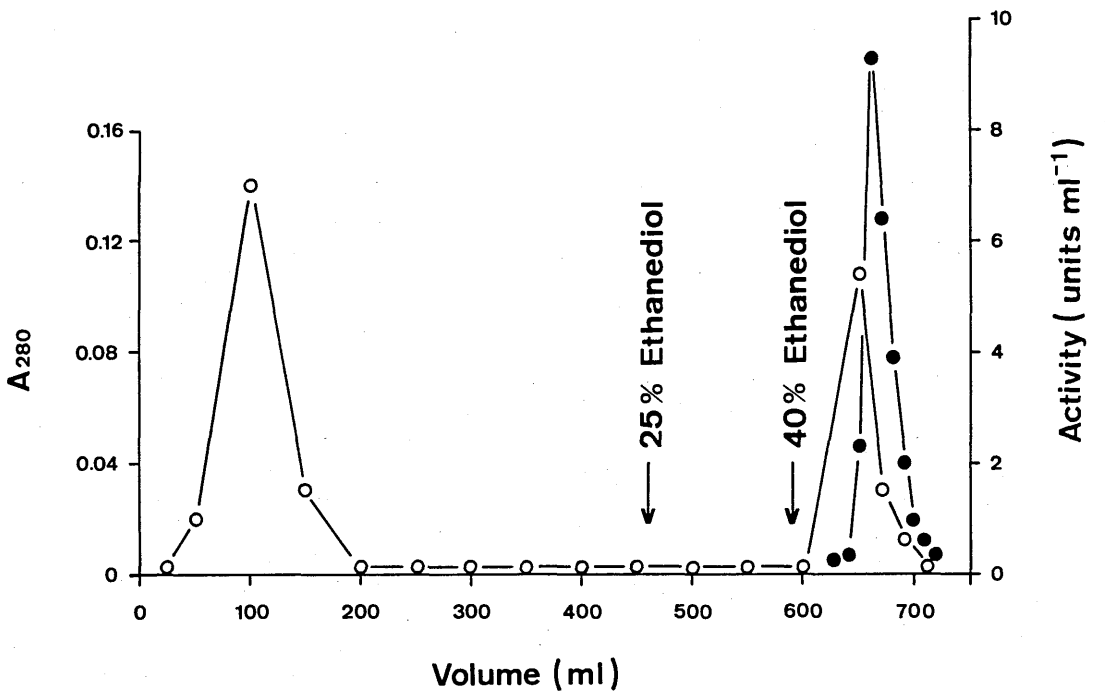


Figure 3.2 Chromatography on Phenyl Sepharose

The DEAE-Sephacel pool (approx. 82 ml) was applied at 30 ml h^{-1} to a Phenyl Sepharose column (10.0 cm x 2.6 cm) pre-equilibrated in buffer A. Once loaded, the column was washed at 40 ml h^{-1} with buffer A until the A_{280} of the effluent returned to the baseline. The column was then washed with 130 ml of buffer A containing 25% (v/v) ethanediol, followed by buffer A containing 40% (v/v) ethanediol which eluted the enzyme.
Enzyme activity (●), A_{280} (○).

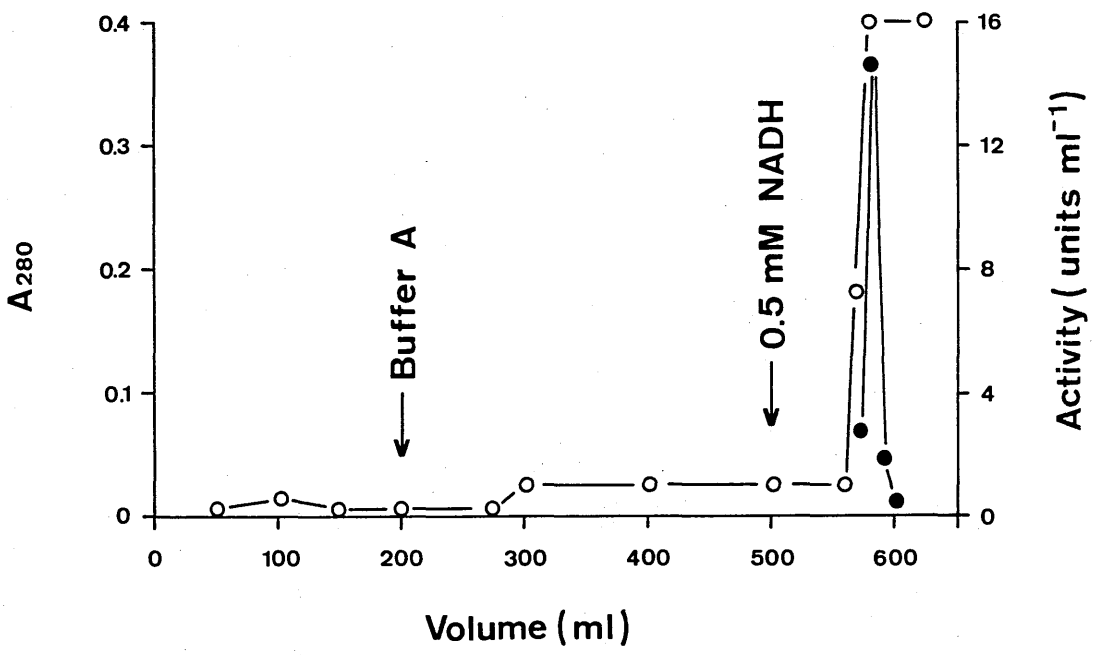


Figure 3.3 Chromatography on Matrex Gel Orange A

The second DEAE-Sephacel pool, dialysed into buffer B [50 mM-potassium phosphate buffer (pH 7.5) containing 1 mM-DTT] was applied to a Matrex Gel Orange A column (15.5 cm x 2.6 cm) pre-equilibrated in buffer B. Once loaded, the pump was stopped for 30 min. Then the column was washed with 200 ml buffer B followed by 300 ml buffer A. The flow was reversed and pure D(—)-mandelate dehydrogenase was eluted with buffer A containing 0.5 mM-NADH. Enzyme activity (●), A_{280} (○).

Table 3.1 Purification of D(—)-mandelate dehydrogenase

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

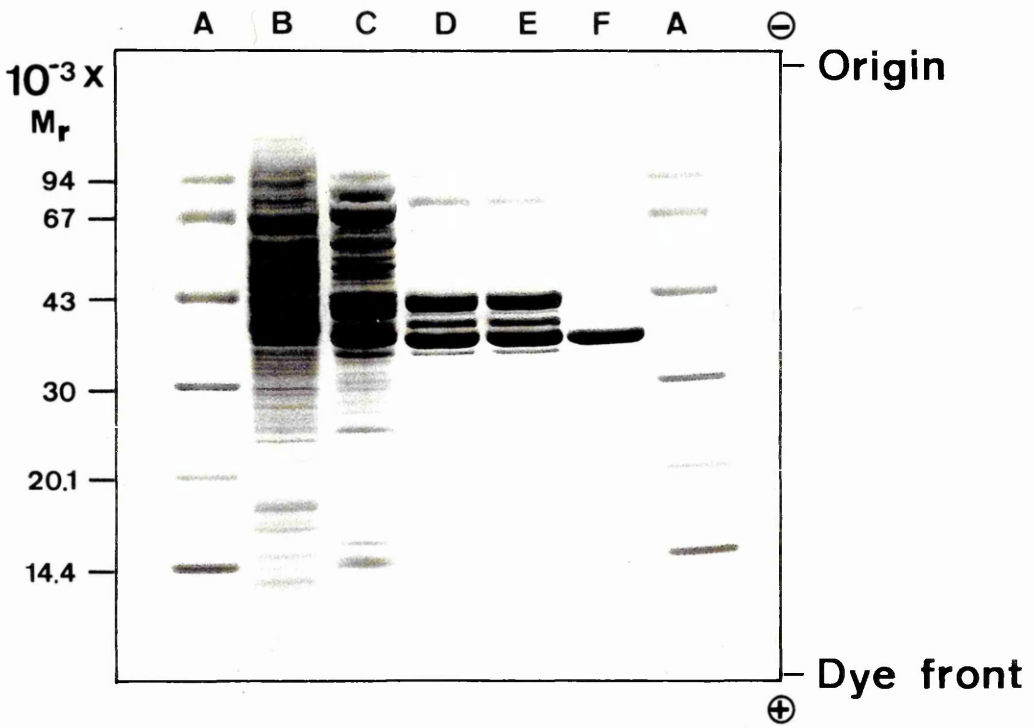


Figure 3.4 Purification of D(—)-mandelate dehydrogenase

The purification was monitored by SDS-PAGE. A 12.5% (w/v) SDS-polyacrylamide gel with a 5.6% stacking gel was run and stained for protein as in Methods 2.10.1 and 2.10.3 (a) respectively. Lanes A, M_r markers; B, 70 μg of the crude extract; C, 40 μg of the DEAE-Sephacel pool; D, 22 μg of the Phenyl Sepharose pool; E, 22 μg of the second DEAE-Sephacel pool; F, 12 μg of the Matrex Gel Orange A pool.

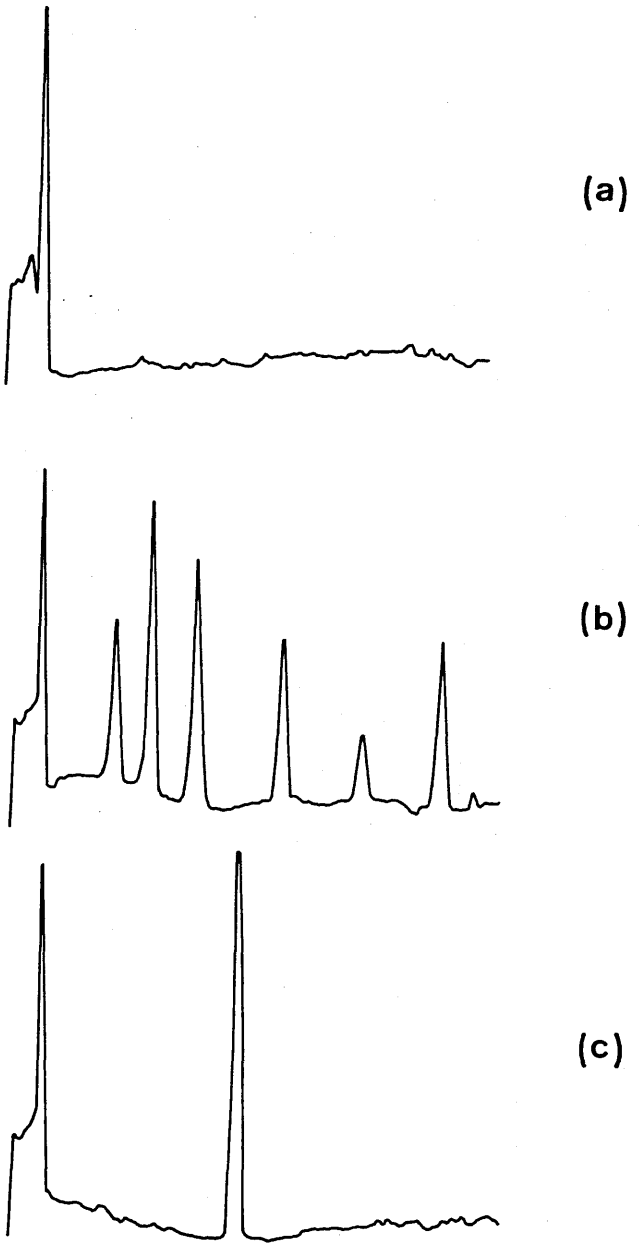


Figure 3.5 Densitometer scans of purified D(—)-mandelate dehydrogenase and M_r markers

Purified D(—)-mandelate dehydrogenase and M_r markers were electrophoresed in 12.5% (w/v) SDS-polyacrylamide gels with 5.6% (w/v) stacking gels (Methods 2.10.1). They were then stained for protein [Methods 2.10.3 (a)] and scanned with a laser densitometer (Methods 2.10.4). (a) Control (no sample loaded); (b) M_r markers (approx. 1 μg of each), from left to right : phosphorylase b (M_r 94 000), BSA (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soya bean trypsin inhibitor (M_r 20 100) and α-lactalbumin (M_r 14 400); (c) pure D(—)-mandelate dehydrogenase (1 μg).

The scans are aligned at the peak marking the start of the resolving gel (far left).

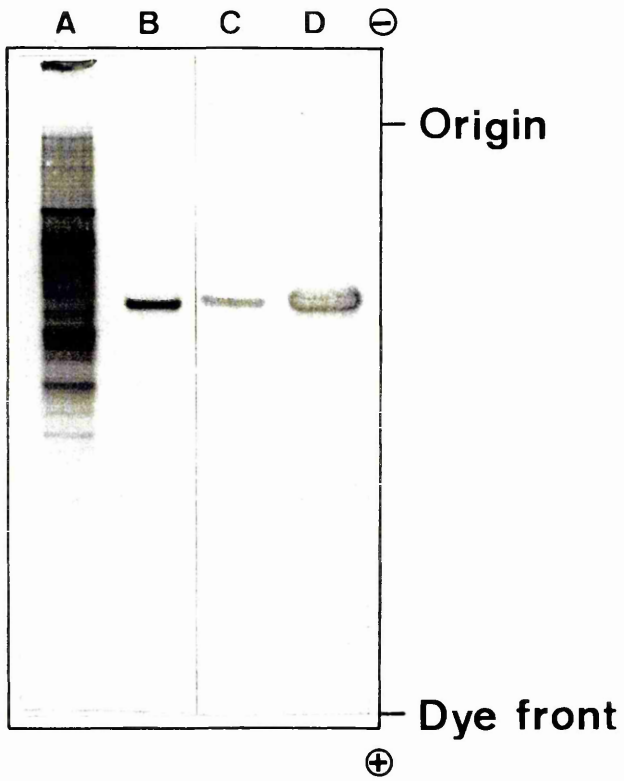


Figure 3.6 Purity of D(—)-mandelate dehydrogenase

The final purity of D(—)-mandelate dehydrogenase was monitored using non-denaturing PAGE. A 7.5% (w/v) polyacrylamide gel with a 5.6% stacking gel was run as in Methods 2.10.2. It was then cut into two and one half stained for protein [Methods 2.10.3 (a)], whilst the other half was stained for D(—)-mandelate dehydrogenase activity [Methods 2.10.3 (b)]. Lanes A, 40 μg of the crude extract stained for protein; B, 2 μg of pure D(—)-mandelate dehydrogenase stained for protein; C, 40 μg of the crude extract stained for activity; D, 2 μg of pure D(—)-mandelate dehydrogenase stained for activity.

3.10 Discussion

A reliable and reproducible method for the purification of mg quantities of homogeneous D(—)-mandelate dehydrogenase has been developed, and the procedure used only three types of chromatographic interaction; anionic exchange, hydrophobic and dye-affinity steps.

The DEAE-Sephacel column proved a useful first step as a result of the enzyme eluting at a relatively low NaCl concentration. The Phenyl Sepharose column also produced significant purification because the enzyme remained bound to the column in the absence of added salt, unlike most of the contaminating proteins which ran straight through. The behaviour of D(—)-mandelate dehydrogenase differs from that of many proteins which can bind to hydrophobic columns only in the presence of high concentrations of salt e.g. NaCl or $(NH_4)_2SO_4$. Although proteins that adsorb strongly to hydrophobic matrices are generally insoluble in water e.g. globulins, membrane-associated proteins and those that precipitate at low ammonium sulphate saturation (Scopes, 1987), D(—)-mandelate dehydrogenase is a soluble enzyme which precipitates at 50-70% ammonium sulphate. Its ability to bind to Phenyl Sepharose in the absence of added salt may therefore represent a high proportion of hydrophobic residues exposed on the protein surface or, alternatively, strong interaction between specific surface residues and the matrix phenyl groups.

Immobilised textile dyes have been used extensively in the purification of $NAD(P)^+$/NAD(P)H-dependent oxidoreductases, as well as other enzymes and proteins. Generally, the dye-affinity step forms a part of the overall purification scheme, as above, although some enzymes have been purified using dye-affinity as the sole type of interaction e.g. *Candida nitratophila* assimilatory nitrate reductase (Hipkin *et al.*, 1986). The success of this type of chromatography is due, in part, to the high degree of specificity between the dye molecule and the enzyme. The ability of cofactors such as $NAD(P)^+$ or NAD(P)H to elute the enzymes from the dye-matrix, and the similarity between the chemical structures of the dyes and natural cofactors suggests the dye interacts with the cofactor binding site. However, other factors such as pH, ionic strength and the presence of divalent cations also affect the binding of enzymes to dye-affinity media (Subramanian, 1984).

The use of Matrex Gel Orange A was central to the purification of D(—)-mandelate dehydrogenase. However, it has been successful in relatively few cases when compared to the number of proteins purified using red and blue dye-ligands (Amicon, 1980). The failure of Blue Sepharose CL-6B or Matrex Gel Red A to yield homogeneous D(—)-mandelate dehydrogenase can be attributed to a lower degree of specificity between the dyes and the proteins present. Blue and red dye-ligands usually bind 30-60% of the proteins in a heterogeneous mixture, whereas Orange A typically binds less than 5% (Amicon, 1980) and, therefore, Orange A may be regarded as being more specific towards the proteins it will bind.

The successful characterization of D(—)-mandelate dehydrogenase depended largely on the ability to produce mg quantities of the enzyme. The purification procedure was scaled up with only minor modification, and it should be possible to scale up further if greater quantities were to be required. The enzyme was also very stable when stored in 50% (v/v) glycerol at -20°C, and therefore frequent purification was unnecessary.

CHAPTER 4

PHYSICAL, CHEMICAL AND KINETIC CHARACTERIZATION OF D(—)-MANDELATE DEHYDROGENASE

4.1 Introduction

A preliminary characterization of the D(—)- and L(+)-mandelate dehydrogenases of *Rhodotorula graminis* strain KGX 39 had previously been reported, but was limited to only those properties which could be determined using crude extract (Durham, 1984). The oxidation of D(—)-mandelate was shown to be mediated by a soluble, NAD^+-dependent enzyme, whereas the oxidation of the L(+)-enantiomer was catalysed by an $NAD(P)^+$-independent enzyme that differed with respect to its intracellular location, substrate and cofactor specificities, heat stability, electrophoretic mobility and pH optimum (Durham, 1984). This was the first clearly reported case of an NAD^+-dependent enzyme being involved in the oxidation of mandelate, and so a more thorough characterization of the enzyme was required if detailed comparisons were to be made with other mandelate dehydrogenases, as well as with other prokaryotic and eukaryotic NAD^+-dependent dehydrogenases. Various physical, chemical and kinetic properties of the purified enzyme from *R. graminis* were determined and are reported in this chapter.

4.2 Physical and chemical characterization

4.2.1 Relative molecular mass

The native M_r , estimated under non-denaturing conditions by gel filtration chromatography using a calibrated FPLC (Pharmacia) Superose 6 column, was 77 200 (76 900, 77 500)(Figure 4.1).

The subunit M_r under denaturing conditions was estimated using calibrated SDS-PAGE gels. Gels were scanned and the electrophoretic mobilities with respect to α-lactalbumin calculated using the equation :

$$R_{\alpha\text{-lactalbumin}} = \text{distance travelled by protein/distance travelled by } \alpha\text{-lactalbumin}$$

A representative standard curve of $R_{\alpha\text{-lactalbumin}}$ against $lg_{10}\ M_r$ is shown in Figure 4.2. The estimated subunit M_r of D(—)-mandelate dehydrogenase was 38 000 ± 1400 (n = 13 batches of enzyme). Comparison of the native and subunit M_r values suggests that D(—)-mandelate dehydrogenase exists as a dimer.

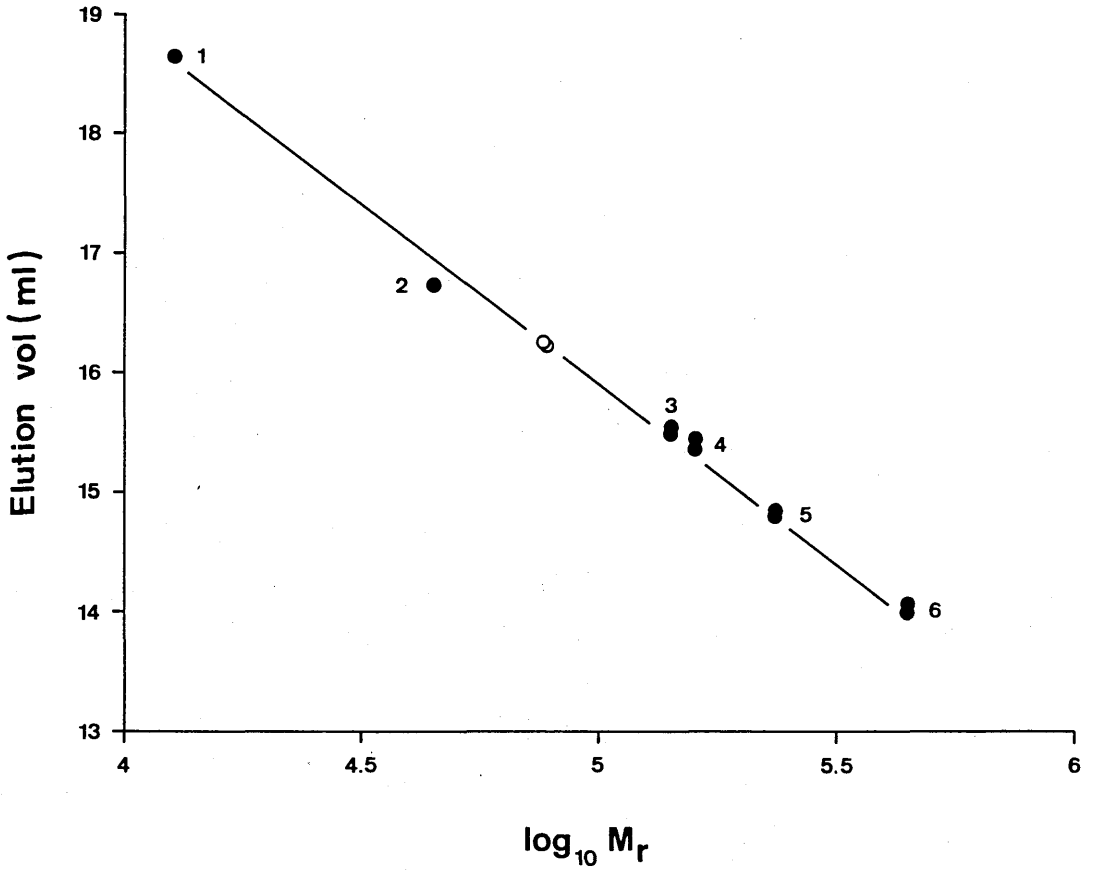


Figure 4.1 Determination of the native M_r of D(—)-mandelate dehydrogenase by gel filtration through a Superose 6 FPLC column

The native M_r of D(—)-mandelate dehydrogenase was estimated under non-denaturing conditions using a Superose 6 (Pharmacia) gel filtration column (30 cm x 1 cm), pre-equilibrated in 50 mM-Tris/HCl buffer (pH 8.0) containing 1 mM-DTT and 100 mM-NaCl. Proteins were eluted at 0.5 ml min^{-1} and the A_{280} of the effluent was monitored (Methods 2.2.5). When D(—)-mandelate dehydrogenase was eluted, fractions (250 μl) were collected and assayed for enzymic activity [Methods 2.4.2 (b)]. Samples were run in duplicate and all data points are shown. Where only one point is given, both values were identical.

(1) 25 μg of cytochrome c (M_r 12 500)

(2) 100 μg of ovalbumin (M_r 45 000)

(3) 50 μg of lactate dehydrogenase (M_r 140 000)

(4) 200 μg of aldolase (M_r 158 000)

(5) 100 μg of pyruvate kinase (M_r 237 000)

(6) 50 μg of ferritin (M_r 450 000)

(○) 50 μg of D(—)-mandelate dehydrogenase

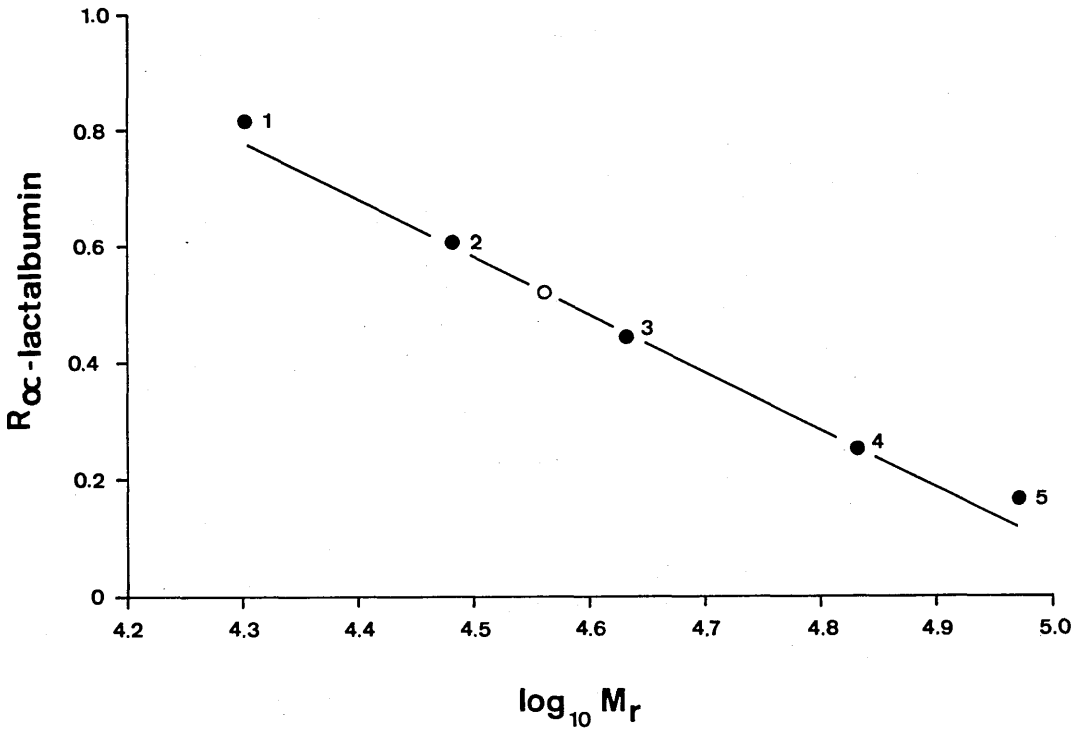


Figure 4.2 Determination of the subunit M_r of D(—)-mandelate dehydrogenase by SDS-polyacrylamide gel electrophoresis

The subunit M_r of D(—)-mandelate dehydrogenase was estimated under denaturing conditions using 12.5% (w/v) SDS-polyacrylamide gels calibrated with proteins of known M_r . Gels were run and stained for protein as in Methods 2.10.1 and 2.10.3 (a) respectively. They were then scanned using a laser densitometer (Methods 2.10.4) and the value of $R_{\alpha\text{-lactalbumin}}$ calculated. A representative standard curve is shown.

(1) soya bean trypsin inhibitor (M_r 20 100)

(2) carbonic anhydrase (M_r 30 000)

(3) ovalbumin (M_r 43 000)

(4) bovine serum albumin (M_r 67 000)

(5) phosphorylase b (M_r 94 000)

(○) D(—)-Mandelate dehydrogenase

4.2.2 Amino acid composition

Table 4.1 shows the amino acid composition of D(—)-mandelate dehydrogenase as the mean value from two independent experiments. Although tryptophan cannot be determined after acid hydrolysis, two residues have so far been identified from sequence analysis (See Tables 4.2 and 5.4). The most notable feature of the amino acid composition was the low number of cysteine residues i.e. only one per subunit. Assuming a subunit M_r of 38 000, the number of residues in the complete polypeptide based on the amino acid composition data is 354 and the average residue M_r is 107.

4.2.3 Amino terminal sequence

The sequence of the first 57 residues from the amino terminus is given in Table 4.2 along with the recovery of each PTH-amino acid. The first residue could not be determined by solid-phase sequencing, as it remained attached to the column via its α-amino group, and lysine gave comparatively poor recoveries as the majority remained attached to the column, via the ε-amino group, after the degradation cycle.

The recent cloning and sequencing of the *mdl*B gene, which encodes the membrane-bound L(+)-mandelate dehydrogenase of *P. putida*, and confirmation of the open reading frame by amino terminal sequencing of the protein electroeluted from polyacrylamide gels, enabled comparison of the *R. graminis* D(—)-mandelate dehydrogenase amino terminal sequence with that of the only other mandelate dehydrogenase for which sequence is available (Tsou *et al.*, 1990; C. A. Fewson, personal communication). However, comparison of the 57 residue yeast sequence with the bacterial amino terminal sequence revealed no obvious homology. Likewise, there was no homology between the *R. graminis* D(—)-mandelate dehydrogenase amino terminal sequence and that of the corresponding sequence of mandelate racemase from *P. putida* (Ransom *et al.*, 1988).

The *R. graminis* D(—)-mandelate dehydrogenase sequence was also compared with the protein sequences in the University of Leeds OWL Composite Database (Release 1.1), which contains all the protein sequences in the NBRF PIR and NEW 16.0, SWISS-PROT 6.0, GenBank 54, NEWAT 86, JIPID and Brookhaven (October 1987) data bases (Akrigg *et al.*, 1988). Although the database contains the amino acid sequences of over 10 000 proteins, including many dehydrogenases, no significant homologies were found. The sequence was later compared with the sequences in the OWL Composite Database (Release 6.0), but again no homologies were found.

4.2.4 Isoelectric point

The isoelectric point of D(—)-mandelate dehydrogenase was determined by chromatofocussing using an FPLC Mono P chromatofocussing column (Pharmacia) (Figure 4.3), and was found to be 5.9 (5.85, 5.95).

Table 4.1 Amino acid composition of D(—)-mandelate dehydrogenase

The amino acid composition of D(—)-mandelate dehydrogenase was determined as described in Methods 2.15.3. The mean values from two independent determinations using separate batches of enzyme are given with individual values in parentheses. The individual values were determined as the mean of 8 replicates, except for valine, leucine, isoleucine, serine, threonine and cysteine (see footnote) which were the mean of 2 replicates. The data were normalized to alanine = 23 nmoles and the approximate number of amino acids per subunit was calculated from the subunit M_r of 38 000.

Amino acid	Mean % (by wt.)	Approx. no of residues per subunit
Ala	7.98 (7.94, 8.01)	43
Val$^{\phi}$	6.00 (5.76, 6.24)	23
Leu$^{\phi}$	11.36 (10.81, 11.90)	38
Ile$^{\phi}$	4.59 (4.41, 4.76)	16
Phe	6.30 (6.26, 6.33)	16
Met	1.21 (1.31, 1.10)	4
Pro	4.38 (4.22, 4.53)	18
Gly	4.15 (4.06, 4.24)	28
Ser$^{\infty}$	4.98 (5.06, 4.90)	22
Thr$^{\infty}$	4.54 (4.59, 4.49)	17
Cys*	0.32 (0.33, 0.31)	1
Tyr	2.83 (3.01, 2.64)	7
Asx	9.94 (10.73, 9.14)	33
Glx	12.15 (12.48, 11.81)	36
Lys	6.28 (6.16, 6.39)	19
Arg	8.25 (8.12, 8.38)	20
His	4.79 (4.74, 4.83)	13
		Total n° = 354

$^{\phi}$ Mean of 96 h values. $^{\infty}$ Extrapolated to zero time. *Determined as cysteic acid.

Table 4.2 The amino terminal sequence of D(—)-mandelate dehydrogenase

The amino terminal sequence of D(—)-mandelate dehydrogenase was determined by Dr J. Keen at the SERC Sequencing Unit, Department of Biochemistry, University of Leeds as described in Methods 2.15.6. Approximately 1 nmole of enzyme was sequenced, and the PTH-amino acid identified at each cycle is given, along with the amount recovered in pmoles.

Cycle	PTH-amino acid	pmole	Cycle	PTH-amino acid	pmole
1	Unk	—	30	Pro	136
2	Arg	952	31	Ala	107
3	Pro	310	32	Asn	92
4	Arg	613	33	Leu	43
5	Val	547	34	Thr*	55
6	Leu	469	35	Thr*	78
7	Leu	612	36	His	77
8	Leu	689	37	Asp	47
9	Gly	443	38	Gly	43
10	Asp	403	39	Phe	68
11	Pro	218	40	Lys?	6
12	Ala	368	41	Gln	69
13	Arg	508	42	Ala	34
14	His	347	43	Leu	71
15	Leu	348	44	Arg	41
16	Asp	227	45	Glu	45
17	Asp	286	46	Lys?	3
18	Leu	298	47	Arg	16
19	Trp	113	48	Tyr	23
20	Ser*	83	49	Gly	32
21	Asp	135	50	Asp	14
22	Phe	174	51	Phe	19
23	Gln	168	52	Unk	—
24	Gln	240	53	Ala	13
25	Lys	17	54	Ile?	9
26	Phe	93	55	Ile	18
27	Glu	73	56	Lys?	4
28	Val	98	57	Lys?	5
29	Ile	106			

Unk - Residue unknown. *Residue confirmed at 313 nm. ? Identification tentative.

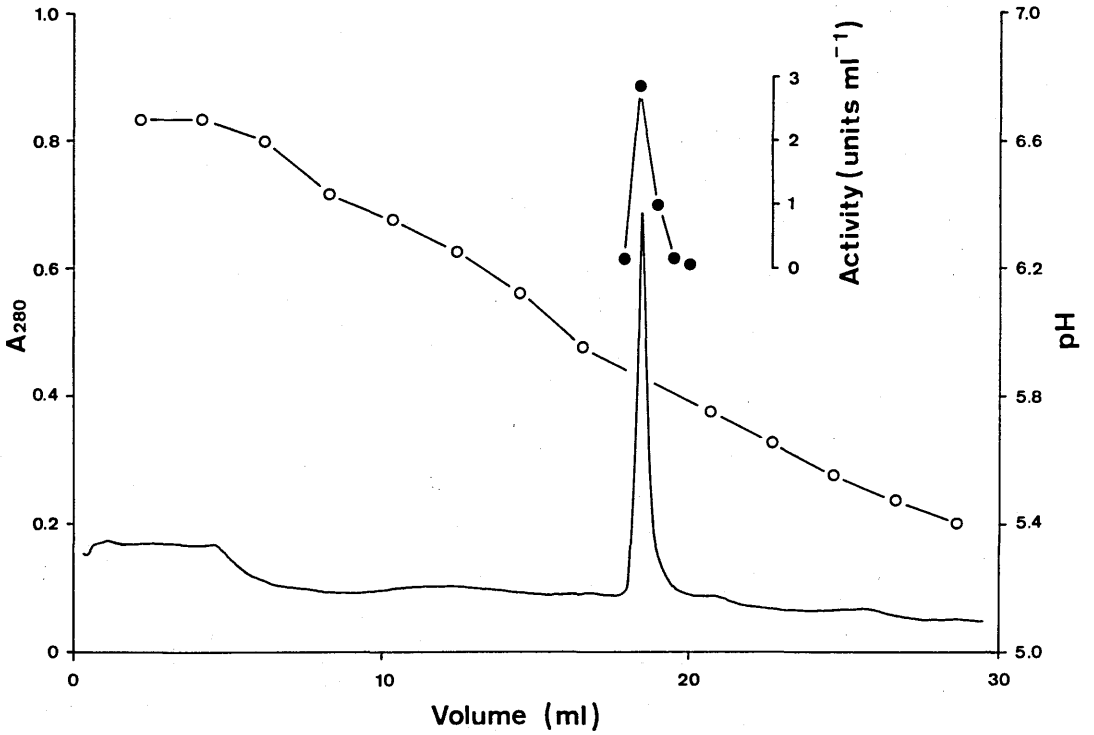


Figure 4.3 Elution profile of D(—)-mandelate dehydrogenase from a Mono P chromatofocussing column

Purified D(—)-mandelate dehydrogenase (100 μg) in 25 mM-Bistris/HCl buffer (pH 6.7) was applied to an FPLC Mono P chromatofocussing column pre-equilibrated in the same buffer. The column was washed with 8 ml of Bistris/HCl buffer before the enzyme was eluted with Polybuffer PB 74 made 1:10 with water and adjusted to pH 3.8. Fractions (1 ml) were collected and assayed for enzymic activity [Methods 2.4.2 (b)]. The A_{280} of the effluent was monitored (Methods 2.2.5) and the pH of each fraction determined (Methods 2.2.1). Enzyme activity (●), pH (○) and A_{280} (——).

4.2.5 Absorption spectrum

The absorption spectrum of D(—)-mandelate dehydrogenase (Figure 4.4) had maxima at about 220 nm and at 280 nm (characteristic of tryptophan absorbance), but no absorbance above 340 nm indicating that the enzyme is neither associated with nor contains bound flavin, haem or cytochrome as prosthetic group.

4.3 Preliminary kinetic characterization

4.3.1 Assay development

In initial experiments designed to measure the rate of D(—)-mandelate oxidation, the enzyme was assayed as described by Durham (1984). The reaction mixture contained 200 mM-Tris/HCl (pH 8.0), 500 μM-NAD^+, 500 μM-D(—)-mandelate and extract. However, the rate was non-linear and the progress curve rapidly reached a plateau as the reaction approached its equilibrium. Therefore, an assay was required which would give linear rates over a 3 to 5 min period and allow accurate measurement of the enzymic activity. Although compounds such as hydrazine and semicarbazide have been used successfully to prevent non-linearity of some enzyme assays e.g. the oxidation of benzyl alcohol to benzaldehyde by benzyl alcohol dehydrogenase (MacKintosh & Fewson, 1988*a*), use of these carbonyl trapping reagents in the D(—)-mandelate dehydrogenase assay proved unsuccessful. The rate of D(—)-mandelate oxidation was finally made linear by assaying the enzyme at a higher pH i.e. 9.5, and at a D(—)-mandelate concentration 10-fold greater than described by Durham (1984). D(—)-Mandelate dehydrogenase catalyses the reversible oxidation of D(—)-mandelate :

$$\text{D(—)-Mandelate} + NAD^+ \;\; <\!\!\longrightarrow \;\; \text{Phenylglyoxylate} + NADH + H^+$$

By increasing the concentration of D(—)-mandelate and decreasing the concentration of H^+, i.e. increasing the pH, the equilibrium was shifted in favour of D(—)-mandelate oxidation and thus allowed accurate measurement of the forward reaction. No such problems were encountered when assaying in the reverse direction (i.e. reduction of phenylglyoxylate) as described in Methods 2.4.2 (c). The reverse assay was more sensitive than the forward assay and generally required 10 times less enzyme.

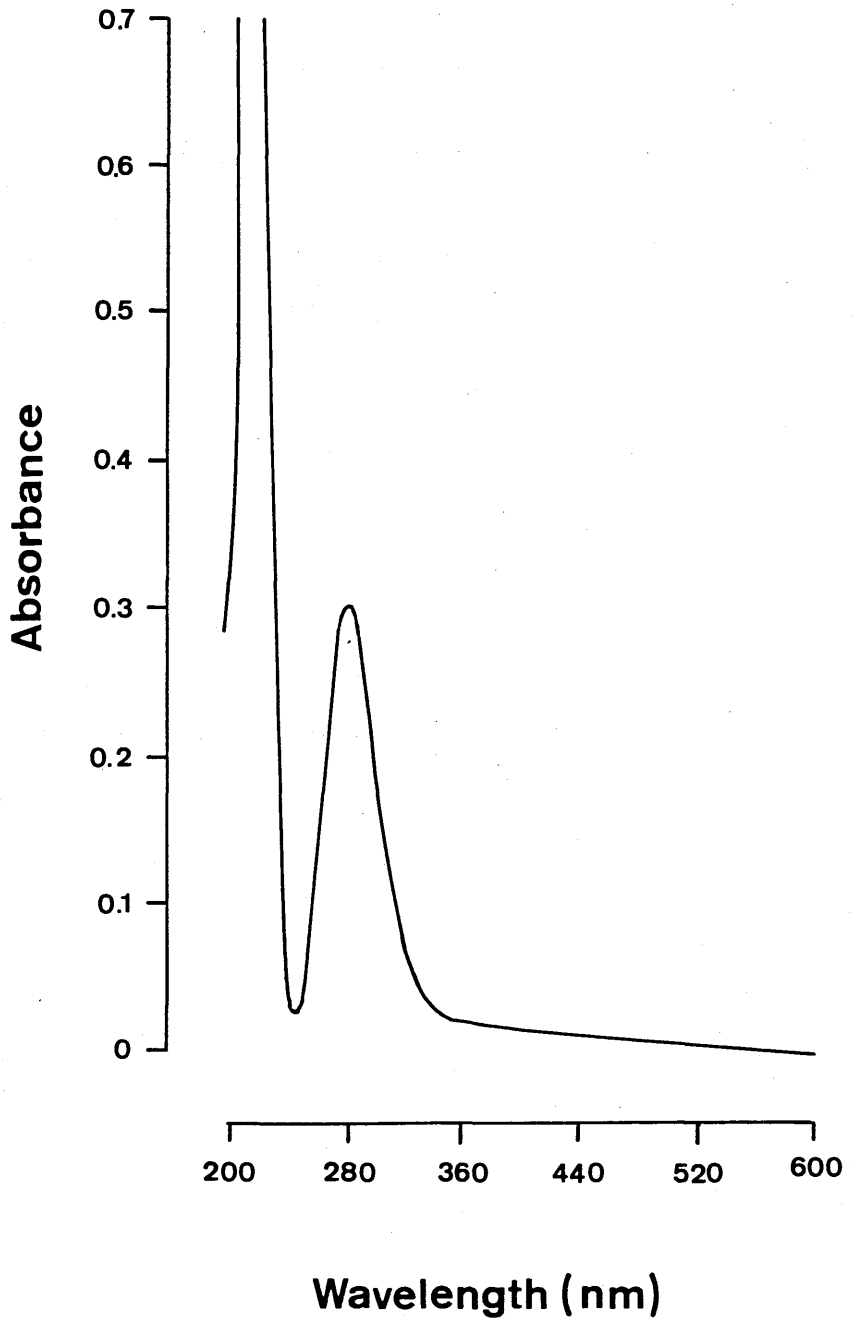


Figure 4.4 Absorption spectrum of D(—)-mandelate dehydrogenase

D(—)-Mandelate dehydrogenase (400 μg) in 50 mM-Tris/HCl (pH 8.0) was scanned in a 1 ml quartz cuvette using a Pye-Unicam SP8-100 spectrophotometer at 2 nm s^{-1}.

4.3.2 The pH optima of D(—)-mandelate dehydrogenase

The pH optimum of the D(—)-mandelate dehydrogenase oxidation reaction was initially determined in appropriate buffers (200 mM) between pH 4.9 and 10.8 [Figure 4.5 (a)]. The enzyme was active over a broad range of pH, but was optimally active between about pH 9 and 10. The enzyme was then assayed in 200 mM-glycine/NaOH buffer between pH 8.1 and 11.1, and in 200 mM-Bicine/NaOH buffer between pH 8.05 and 9.89. Again the enzyme was optimally active between pH 9 and 10, but showed maximal activity at pH 9.5 in both cases.

The pH optimum of the reduction reaction was determined in appropriate buffers (200 mM) between pH 4.6 and 9.6 [Figure 4.5 (b)]. The enzyme catalysed the reduction reaction over a broad range of pH, but was maximally active at pH 5.85 in 200 mM-potassium phosphate/NaOH buffer.

All assays used for kinetic characterization were carried out at the respective pH optima for the oxidation and reduction reactions.

4.3.3 Assay reproducibility and linearity with respect to protein concentration

The reproducibility of the D(—)-mandelate dehydrogenase assay (forward/oxidation reaction) was determined by assaying ten identical amounts of enzyme as in Methods 2.4.2 (b). The standard deviation as a percentage of the mean value was ± 2.8% in one experiment and ± 1.6% in another, indicating that the assay was reproducible.

The rate of D(—)-mandelate oxidation was proportional to the protein concentration, up to the maximum measured concentration of 400 ng ml^{-1}. Assays designed to measure the rate of D(—)-mandelate oxidation contained less than this amount, unless otherwise stated.

4.3.4 Reaction stoichelometry and equilibrium constant

D(—)-Mandelate dehydrogenase catalyses the oxidation of D(—)-mandelate and the reduction of phenylglyoxylate. The reaction stoicheiometry and apparent equilibrium constant (K'_{eq}) were determined by following the progress curve of the reaction. The concentration of substrates and products were presumed to be at equilibrium when the curve reached a plateau. Four progress curves, starting with different NAD^+ and D(—)-mandelate concentrations, are shown in Figure 4.6.

The concentration of NADH at equilibrium was determined from the change in A_{340} [Methods 2.5 (a)]; the concentration of NAD^+ at equilibrium was calculated by deducting the concentration of NADH from the concentration of NAD^+ initially added. The initial amount of NAD^+ added was determined enzymically as described in Methods 2.5 (b).

An HPLC system was developed to measure the concentrations of D(—)-mandelate and phenylglyoxylate in assay mixtures (Methods 2.6). The calibration curves for D(—)-mandelate and phenylglyoxylate are shown in Figures 4.7 and 4.8 respectively. The absorbance of phenylglyoxylate at 210 nm (i.e. the monitoring

wavelength) was approximately 10-fold greater than for D(—)-mandelate and the phenylglyoxylate calibration curve therefore covered the range 0-5 nmoles, whereas the D(—)-mandelate curve covered the range 0-50 nmoles. In each case the lines of best fit were linear, although there was a greater degree of scatter amongst replicates at the higher D(—)-mandelate concentrations.

The concentrations of D(—)-mandelate, NAD^+, phenylglyoxylate and NADH at equilibrium (Table 4.3) indicate that D(—)-mandelate dehydrogenase produced approximately equimolar amounts of phenylglyoxylate and NADH. The enzyme therefore catalyses a reaction with a stoicheiometry of 1 mole of D(—)-mandelate oxidised per mole of NAD^+ reduced. The apparent equilibrium constant for each of the 4 experiments was calculated using the equation :

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

The mean equilibrium constant was 1.59×10^{-11} M at pH 9.2.
The standard free energy change of the reaction was calculated using the equation :

$$\text{Standard free energy change } \Delta G^{o'} = -RT \ln K'_{eq}$$

where R is the gas constant = 8.303 J $deg.^{-1}$ mol^{-1}, and T is the temperature = 300 K.

The standard free energy for the reaction was + 61.9 KJ mol^{-1}, and the reduction of phenylglyoxylate and oxidation of NADH is therefore thermodynamically favoured at this pH.

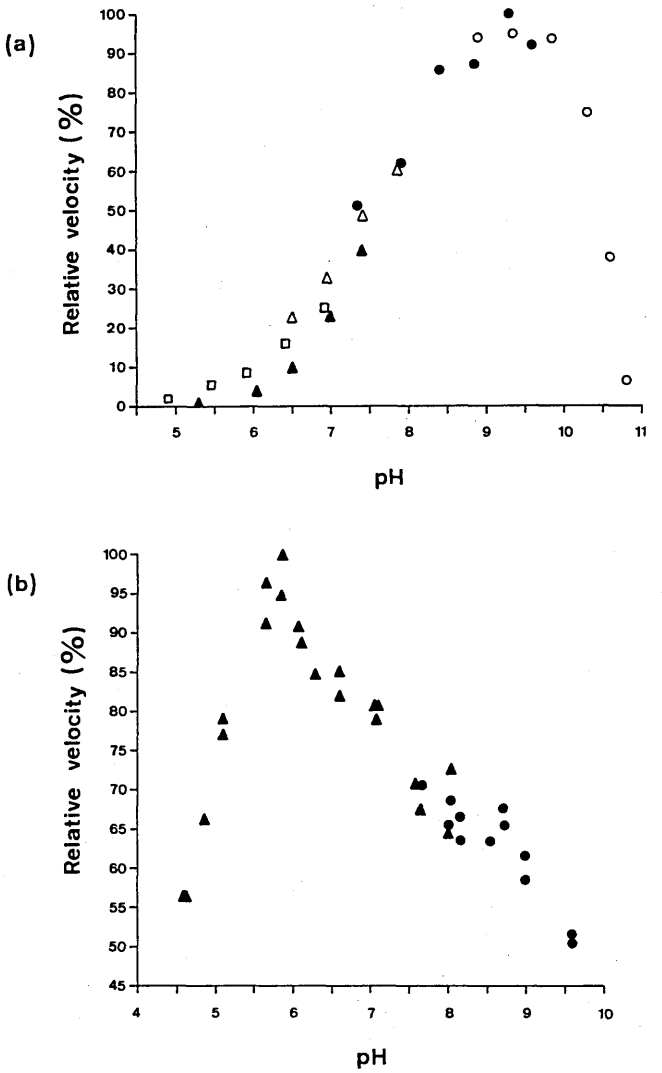


Figure 4.5 Effect of pH on the oxidation and reduction reactions of D(—)-mandelate dehydrogenase

(a) The oxidation reaction was measured in a reaction mixture (1 ml) containing 200 mM-buffer, 1 mM-NAD^+, 5 mM-D(—)-mandelate and D(—)-mandelate dehydrogenase (63 ng or 127 ng). The pH of the mixture was determined shortly after the assay, as described in Methods 2.2.1. The maximum (100%) velocity was 125 units $(mg\ protein)^{-1}$.

(□) Mes/NaOH buffer, pH 4.9-6.9

(▲) Potassium phosphate buffer, pH 5.3 - 7.4

(△) Mops/NaOH buffer, pH 6.5 - 7.85

(●) Bicine/NaOH buffer, pH 7.35 - 9.6

(○) Glycine/NaOH buffer, pH 8.9 - 10.8

(b) The reduction reaction was measured in a reaction mixture (1 ml) containing 200 mM-buffer, 1 mM-phenylglyoxylate, 200 μM-NADH and D(—)-mandelate dehydrogenase (6 ng). The pH of the mixture was determined shortly after the assay, as described in Methods 2.2.1. The maximum (100%) velocity was 497 units $(mg\ protein)^{-1}$.

(▲) Potassium phosphate buffer, pH 4.6 - 8.0

(●) Bicine/NaOH buffer, pH 7.65 - 9.6

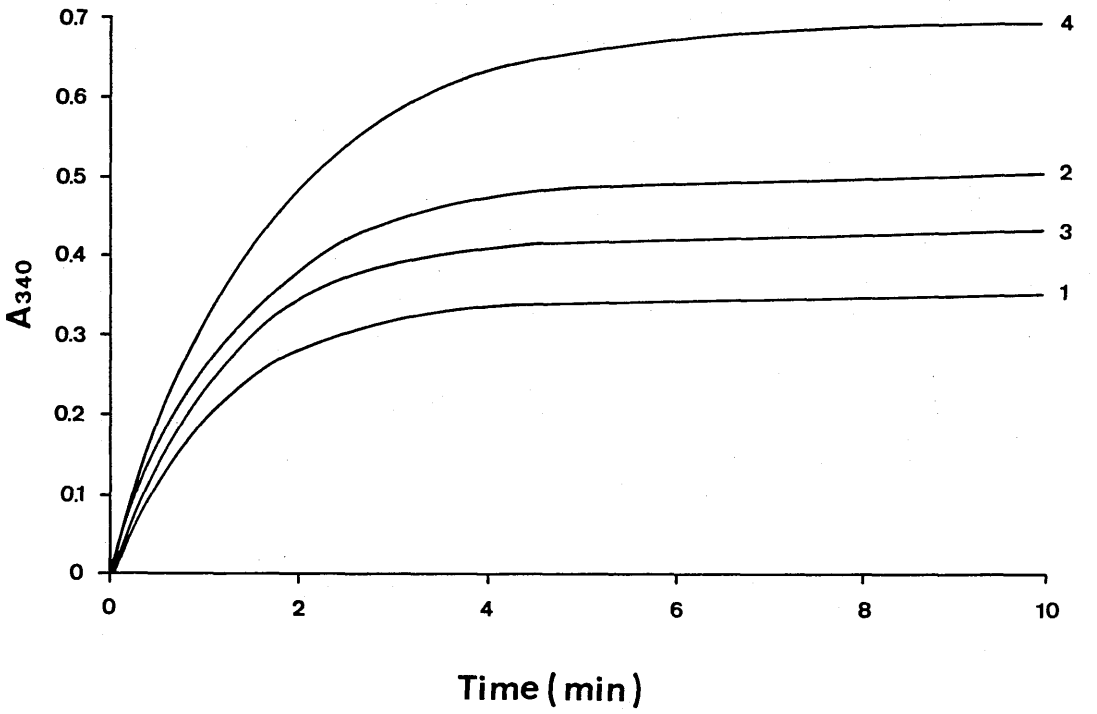


Figure 4.6 Progress curves of the D(—)-mandelate dehydrogenase oxidation (forward) reaction

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

Reaction mixture 1 : 1.1 mM-D(—)-mandelate + 169 μM-NAD^+

Reaction mixture 2 : 1.1 mM-D(—)-mandelate + 338 μM-NAD^+

Reaction mixture 3 : 2.2 mM-D(—)-mandelate + 169 μM-NAD^+

Reaction mixture 4 : 2.2 mM-D(—)-mandelate + 338 μM-NAD^+

Each reaction mixture contained 774 ng of enzyme in the 1 ml reaction mixture.

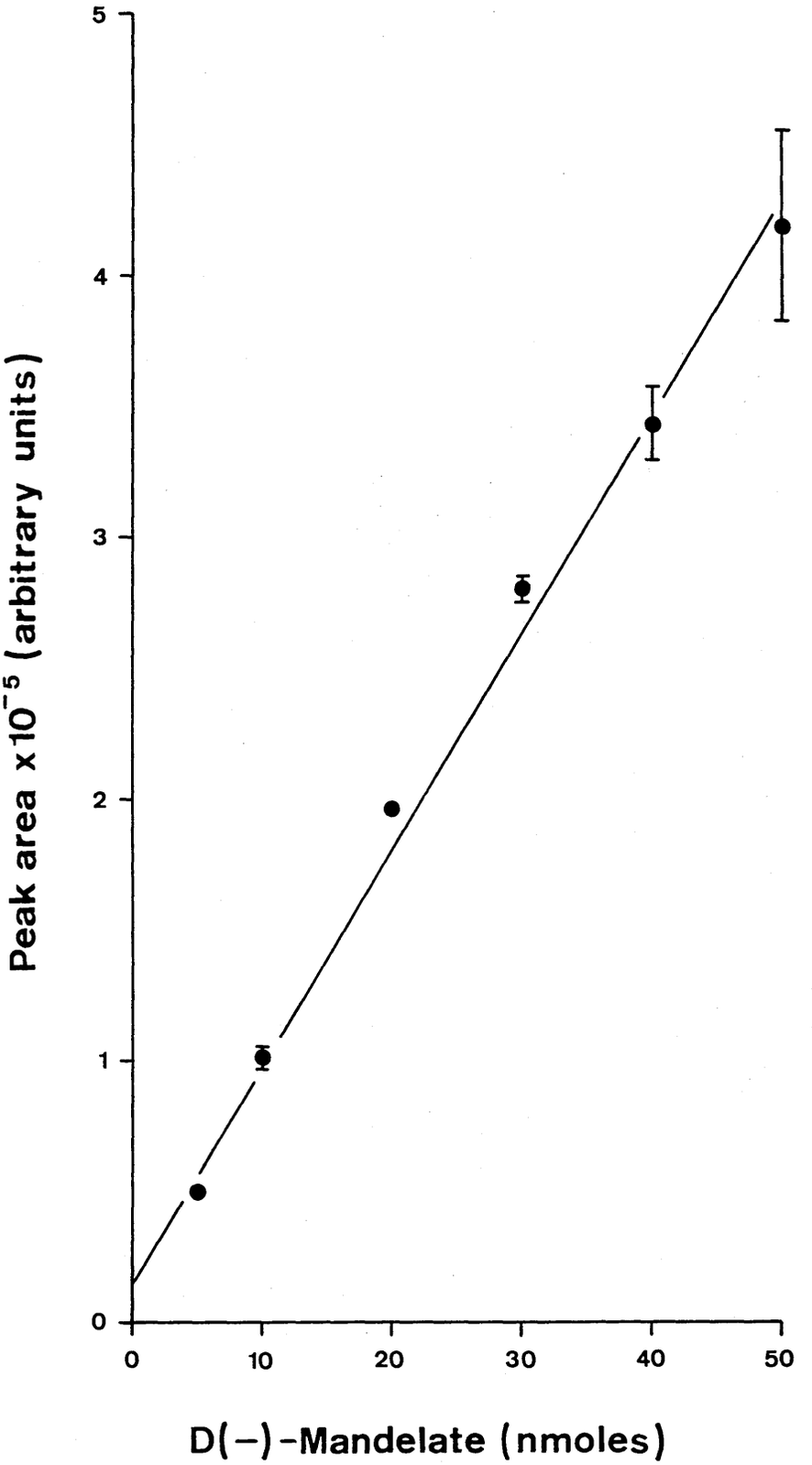


Figure 4.7 Calibration curve for the estimation of D(—)-mandelate by HPLC

Known amounts of D(—)-mandelate were injected onto a C_4 RP-304 reverse-phase column and eluted in 0.1% TFA at 1 ml min^{-1}. The effluent was monitored at 210 nm and the peak areas determined by an on-line integrator (Methods 2.6). Two to four injections at each D(—)-mandelate concentration were made and where only one point is given, the replicates were identical. Error bars indicate ± 1 standard deviation about the mean.

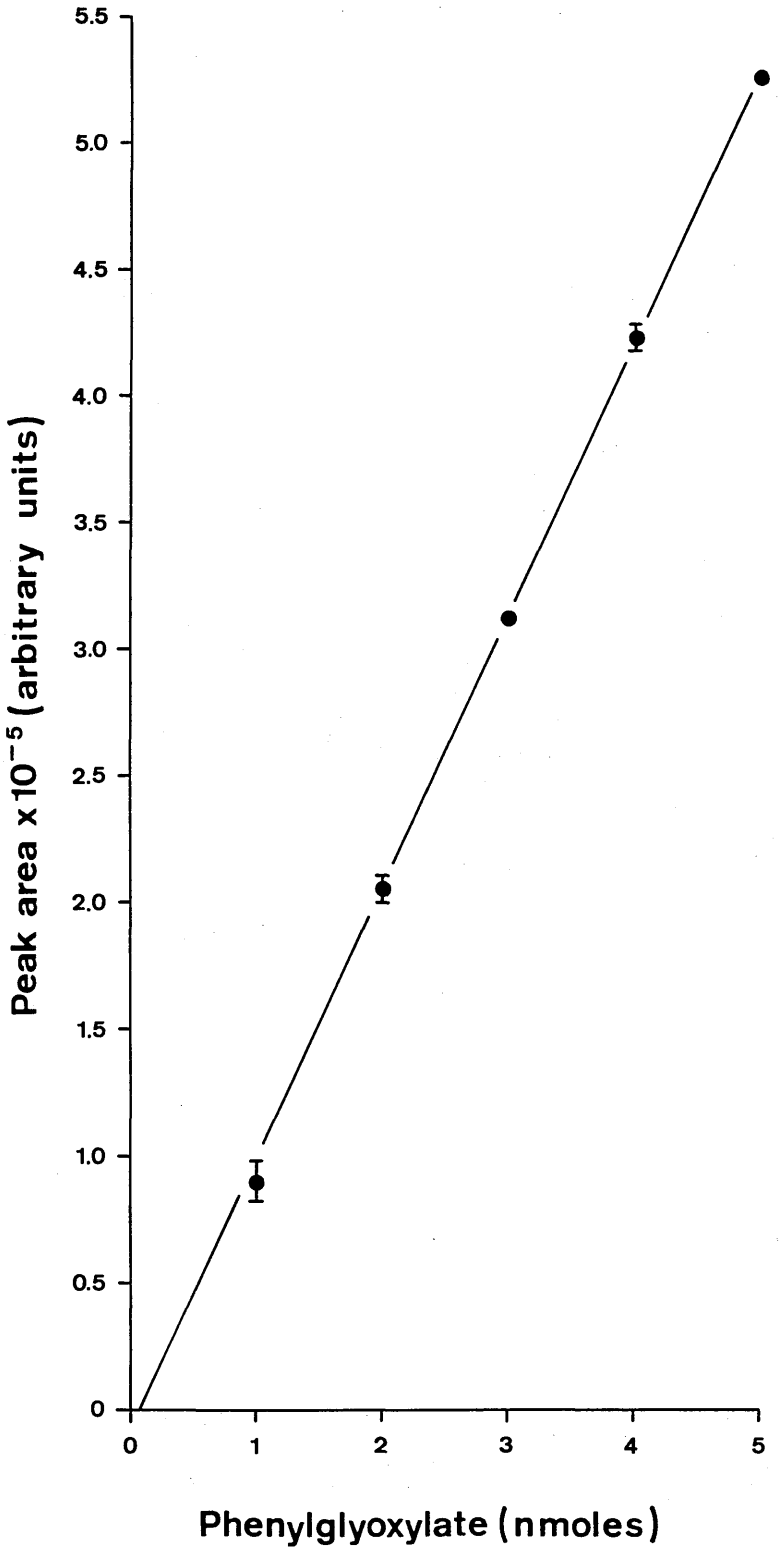


Figure 4.8 Calibration curve for the estimation of phenylglyoxylate by HPLC

Known amounts of phenylglyoxylate were injected onto a C_4 RP-304 reverse-phase column and eluted in 0.1% TFA at 1 ml min^{-1}. The effluent was monitored at 210 nm and the peak areas determined by an on-line integrator (Methods 2.6). Two to four injections at each phenylglyoxylate concentration were made and where only one point is given, replicates were identical. Error bars indicate ± 1 standard deviation about the mean.

Table 4.3 Concentrations of D(—)-mandelate, NAD^+, phenylglyoxylate and NADH at equilibrium

The oxidation (forward) reaction of the enzyme was measured in 200 mM-Bicine/NaOH buffer (pH 9.2) in the presence of the following starting concentrations of D(—)-mandelate and NAD^+. The results refer to the experiment illustrated in Figure 4.6.

Reaction mixture 1 : 1.1 mM-D(—)-mandelate + 169 μM-NAD^+

Reaction mixture 2 : 1.1 mM-D(—)-mandelate + 338 μM-NAD^+

Reaction mixture 3 : 2.2 mM-D(—)-mandelate + 169 μM-NAD^+

Reaction mixture 4 : 2.2 mM-D(—)-mandelate + 338 μM-NAD^+

Reaction mixture	Concentration at equilibrium (μM)				Apparent K_{eq}
	D(—)-mandelate	NAD^+	phenylglyoxylate	NADH	(M)
1	925	113	53.7	56.0	1.82×10^{-11}
2	920	257	75.0	80.4	1.43×10^{-11}
3	2150	100	76.2	69.0	1.53×10^{-11}
4	1980	227	102.5	110.3	1.58×10^{-11}

4.3.5 Cofactor specificity

(a) Electron acceptor specificity

D(—)-Mandelate dehydrogenase is an NAD^+-dependent enzyme and could neither reduce $NADP^+$ (in the forward reaction) nor oxidise NADPH (in the reverse reaction) at detectable rates when assayed as in Methods 2.4.2 (b) and (c), with $NADP^+$ (1 mM) and NADPH (200 μM) replacing the NAD^+ and NADH respectively. The enzyme was also unable to use the dye DCIP (53 μM) or DCIP (53 μM) plus PMS (1 mM) as electron acceptors when assayed as in Methods 2.4.2 (b), with the dyes replacing the NAD^+.

(b) Stereospecificity of hydride transfer

The stereospecificity of hydride transfer was determined by measuring the ratio of label (^3H) incorporated into NAD^+ and D(—)-mandelate after the enzyme had been incubated in the presence of phenylglyoxylate and either pro-*S* ^3H-NADH or pro-*R* ^3H-NADH (Methods 2.14).

The stereospecificity of the enzyme was designated according to the following criteria : starting with pro-*S* ^3H-NADH, if the enzyme transferred the pro-*S* hydrogen from NADH to phenylglyoxylate, then all of the label would be incorporated into D(—)-mandelate, but if it transferred the pro-*R* hydrogen then the label would remain in the NAD^+. On the other hand, starting with pro-*R* ^3H-NADH, if the enzyme transferred the pro-*R* hydrogen from NADH to phenylglyoxylate, then all of the label would be incorporated into D(—)-mandelate, but if it transferred the pro-*S* hydrogen then the label would remain in the NAD^+.

When D(—)-mandelate dehydrogenase was incubated with phenylglyoxylate and pro-*S* ^3H-NADH, the ratio of total counts incorporated into the resultant NAD^+ and D(—)-mandelate was 95.7% : 4.3% respectively, indicating that the enzyme transferred the pro-*R* hydrogen. This result was confirmed when the enzyme was incubated with phenylglyoxylate and pro-*R* ^3H-NADH, where the ratio of total counts incorporated into the NAD^+ and D(—)-mandelate was 2.4% : 97.6% respectively. Final confirmation that the label from the pro-*R* ^3H-NADH was covalently incorporated into D(—)-mandelate was obtained after recrystallization. The tritiated D(—)-mandelate (44 nmol, 0.7 μCi) purified by HPLC [Methods 2.14 (c)], was diluted with non-radiolabelled D(—)-mandelic acid (678 mg, 4.5 mmol) and recrystallized from toluene. The needle-shaped crystals were washed thoroughly with ice-cold pentane and then lyophilized. This procedure was repeated twice and the specific activities of the D(—)-mandelic acid after recrystallization were 131 μCi mol^{-1} and 130 μCi mol^{-1} i.e. it had recrystallized with a constant specific activity.

4.3.6 Substrate and inhibitor specificity

D(—)-Mandelate dehydrogenase showed absolute stereospecificity for its substrate and could not oxidise L(+)-mandelate, even at a concentration of 70 mM. However, the L(+)-enantiomer was an inhibitor of the enzyme. In order to determine the nature of this inhibition, the enzyme was assayed at various concentrations of D(—)-mandelate at a series of fixed L(+)-mandelate concentrations. The effect of increasing the L(+)-mandelate concentration on the primary double-reciprocal plots {1/*V* against 1/[D(—)-mandelate], Figure 4.9 (a)} was indicative of competitive inhibition i.e. the slopes increased whilst leaving the ordinate intercepts essentially unchanged. The apparent K_i value for L(+)-mandelate was obtained by plotting the slopes from the primary plots against the L(+)-mandelate concentration [Figure 4.9 (b)], and was found to be 8.2 mM.

The ability of D(—)-mandelate dehydrogenase to use a number of ring-substituted mandelates and mandelate analogues as substrates was tested. However, velocity values could not be determined for two reasons. (a) Some of the corresponding substituted phenylglyoxylates absorbed strongly at 340 nm (i.e. the monitoring wavelength), unlike phenylglyoxylate which had negligible absorbance at this wavelength. The molar absorption coefficients at 340 nm of these compounds could, in principle, be used to determine correction factors (as in similar work by MacKintosh & Fewson, 1988*b*) but this was not possible as these compounds were not commercially available, and it was not considered worthwhile synthesizing them. (b) Most of the compounds were tested as racemic mixtures and it is possible that some of the L-enantiomers were inhibitory. Compounds which were substrates in the oxidation and reduction reactions are given in Table 4.4.

Other compounds which were not substrates for the enzyme but which inhibited activity are given in Tables 4.5 and 4.6. The enzyme was assayed in the presence of inhibitor at the calculated K_m value for D(—)-mandelate or the apparent K_m value for phenylglyoxylate.

D(+)-Malate, citrate and glyoxylate at 10 mM were neither substrates nor inhibitors of activity.

The effect of possible metabolic inhibitors on D(—)-mandelate dehydrogenase activity was also tested. The enzyme was neither inhibited nor stimulated by 1 mM concentrations of benzaldehyde, benzoate, succinate, glucose, ATP/Mg_2^+, ADP/Mg_2^+ or acetyl-CoA when the enzyme was assayed as in Methods 2.4.2 (b), suggesting that enzymic activity is not regulated *in vivo* by these compounds. However, the enzyme was apparently inhibited 44% by 0.5 mM-phenylglyoxylate and was progressively inhibited by increasing the concentration until, at 10 mM-phenylglyoxylate, the activity was virtually abolished (96% inhibition). As D(—)-mandelate dehydrogenase catalyses a reversible reaction and the equilibrium strongly favours the reverse reaction (Section 4.3.4), this is likely to be due to an equilibrium effect rather than inhibition of activity caused by the binding of phenylglyoxylate at a possible regulatory site.

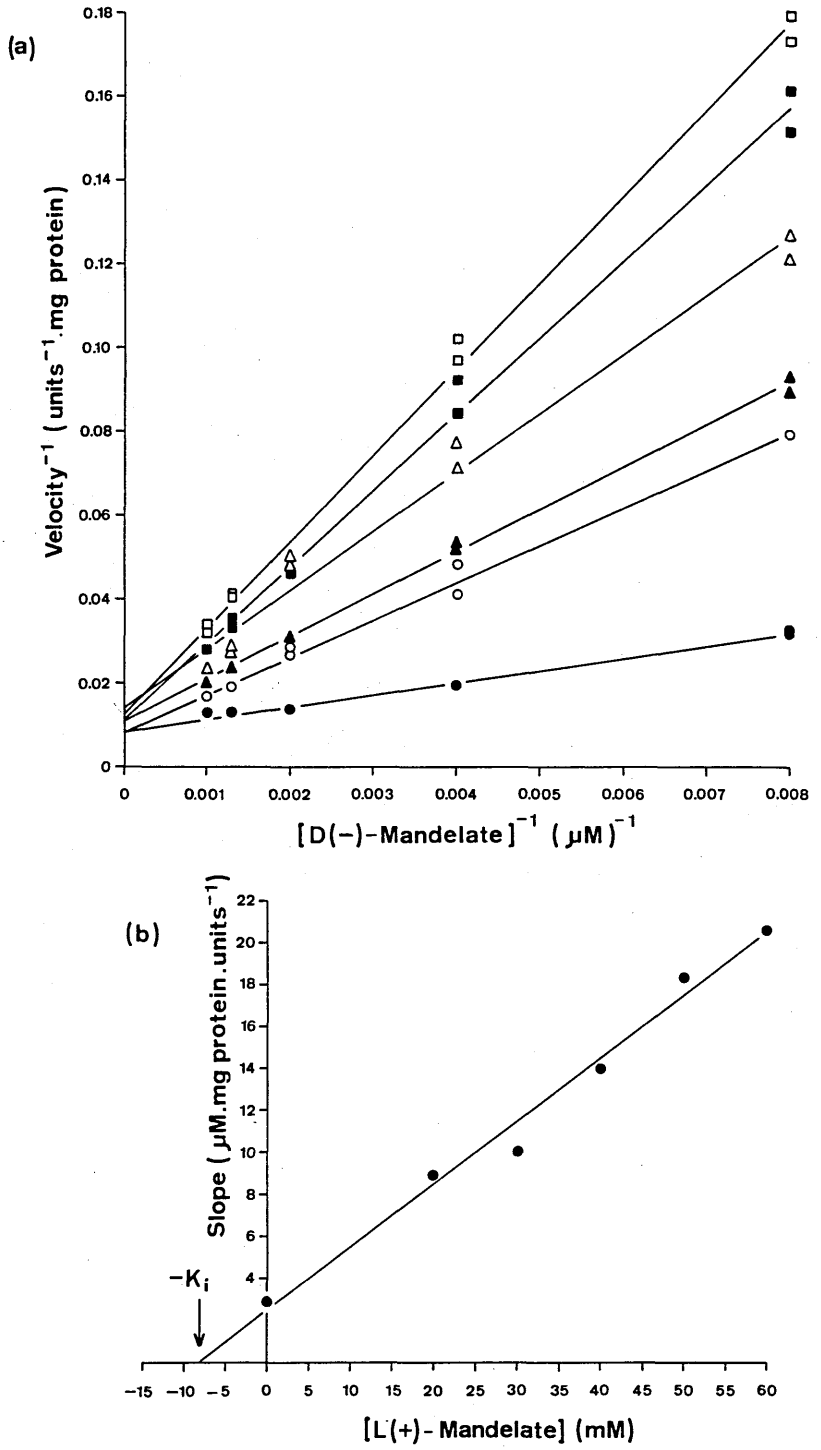


Figure 4.9 Competitive inhibition of the D(—)-mandelate dehydrogenase oxidation reaction by L(+)-mandelate

(a) Primary double-reciprocal plots of the initial velocities against D(—)-mandelate concentration at a series of fixed L(+)-mandelate concentrations :

(●), none; (○), 20 mM; (▲), 30 mM; (△), 40 mM; (■), 50 mM; (□) 60 mM. Enzyme (154 ng) was assayed in 200 mM-Bicine/NaOH buffer (pH 9.5), containing 1 mM-NAD^+. Assays were carried out in duplicate and where only one point is given, the replicates were identical.

(b) Replot of slopes from Figure 4.9 (a) as a function of L(+)-mandelate concentration.

Table 4.4 Substrates of D(—)-mandelate dehydrogenase

Although velocity values cannot be given (Section 4.3.6), the observed rates of change in A_{340} with time, as a percentage of the value obtained with D(—)-mandelate or phenylglyoxylate as substrate, are given. The oxidation reaction was assayed in 200 mM-Bicine/NaOH buffer (pH 9.5) containing enzyme (154 ng or 15.4 ng for assays with D,L-4-hydroxymandelate and D,L-4-hydroxy-3-methoxymandelate), 1 mM-NAD^+ and substrate. The reduction reaction was measured in 200 mM-potassium phosphate/NaOH buffer (pH 5.85) containing enzyme (13.4 ng), 190 μM-NADH and substrate. Values in parentheses represent values from two individual experiments, except for thiopheneglyoxylate where the value in parentheses represents the number of individual experiments.

Oxidation reaction

Compound	Percentage rate of change A_{340}
1 mM-D,L-3-Hydroxymandelate	79.4 (78.4, 80.3)
1 mM-D,L-4-Hydroxymandelate	1322 (1289, 1355)
1 mM-D,L-4-Hydroxy-3-methoxymandelate	705 (698, 711)
10 mM-D,L-3-Hydroxy-4-methoxymandelate	23.4 (23.0, 23.8)
10 mM-D,L-2-Methoxymandelate	7.0 (7.3, 6.6)
1 mM-D,L-4-Fluoromandelate	69.7 (69.3, 70.0)
1 mM-D,L-4-Chloromandelate	26.7 (26.7, 26.7)
1 mM-D,L-4-Bromomandelate	14.8 (14.4, 15.2)
10 mM-D(—)-Hexahydromandelate	13.4 (13.0, 13.7)

Reduction reaction

Compound	Percentage rate of change A_{340}
1 mM-Phenylpyruvate	13.2 (13.2, 13.2)
1 mM-Thiopheneglyoxylate	10.1 ± 1.5 (5)

Table 4.5 Inhibition of the oxidation reaction of D(—)-mandelate dehydrogenase by compounds which are not substrates

The enzyme was assayed in 200 mM-Bicine/NaOH buffer (pH 9.5) containing enzyme (154 ng), 1 mM-NAD^+, 319 μM-D(—)-mandelate and inhibitor. The initial velocity in the absence of inhibitor was taken as the 100% value. Values in parentheses represent results from two individual experiments.

Compound	Percentage inhibition
10 mM-D(—)-Lactate	17.4 (17.4, 17.4)
10 mM-D,L-Phenyllactate	47.1 (46.4, 47.9)
5 mM-D,L-4-Hydroxyphenyllactate	44.2 (43.5, 45.0)
10 mM-D,L-2-Hydroxyvalerate	67.4 (68.2, 66.7)
10 mM-D,L-2-Hydroxyisovalerate	31.9 (30.5, 33.4)
1 mM-D,L-2-Hydroxycaproate	63.8 (63.8, 63.8)
1 mM-D,L-2-Hydroxyisocaproate	55.1 (53.7, 56.6)
10 mM-Oxalate	63.1 (63.8, 62.4)

Table 4.6 Inhibition of the reduction reaction of D(—)-mandelate dehydrogenase by compounds which are not substrates

The enzyme was assayed in 200 mM-potassium phosphate/NaOH buffer (pH 5.85) containing enzyme (13.4 ng), 200 μM-NADH, 51.2 μM-phenylglyoxylate and inhibitor. The initial velocity in the absence of the inhibitor was taken as the 100% value. Values in parentheses represent results from two individual experiments.

Compound	Percentage inhibition
10 mM-Pyruvate	17.4 (17.4, 17.4)
10 mM-Oxaloacetate	38.5 (35.8, 41.3)
10 mM-Oxamate	77.1 (78.0, 76.2)
10 mM-2-Oxoglutarate	44.1 (43.2, 45.0)
10 mM-2-Oxovalerate	43.1 (41.3, 45.0)
10 mM-2-Oxoisovalerate	44.1 (43.2, 45.0)
1 mM-2-Oxoisocaproate	40.4 (43.2, 37.7)
2.5 mM-2-Oxooctanoate	28.5 (30.3, 26.7)
5 mM-2-Oxo-3-methylvalerate	54.1 (54.1, 54.1)

4.3.7 Effects of salts, metal ions and metal chelators

D(—)-Mandelate dehydrogenase activity was neither stimulated nor inhibited (after a 5 min pre-incubation at 27°C) by 1 mM concentrations of NaCl, Na_2SO_4, KCl, $MgCl_2$, NH_4Cl, $CaCl_2$, $ZnCl_2$, $CoCl_2$, $FeCl_3$, KH_2PO_4, NaH_2PO_4, $MnSO_4$, $MnCl_2$ or $CuSO_4$.

Enzyme activity was also not inhibited (after a 5 min pre-incubation at 27°C) by the following metal-chelating agents each at 1 mM concentration : EDTA, diethyldithiocarbamic acid, bathophenanthroline disulphonic acid, dihydroxybenzene disulphonic acid, pyrazole, 8-hydroxyquinilone or 2,2′-dipyridyl, indicating that the enzyme has no absolute requirement for salts or metal ions.

4.3.8 Effects of thiol reagents

D(—)-Mandelate dehydrogenase was inhibited by 10 mM-*N*-ethylmaleimide, resulting in a 90% loss of activity after a 5 h incubation at 27°C. However, there was little or no inhibition of enzyme activity by 5 mM-4-(chloromercuri)benzoate, 10 mM-iodoacetate, 10 mM-iodoacetamide or 6.65 mM-5,5′-dithiobis(2-nitrobenzoic acid), even after a 6 h pre-incubation at 27°C.

4.4 Steady state kinetics

4.4.1 Kinetic coefficients of the oxidation reaction of D(—)-mandelate dehydrogenase with D(—)-mandelate and NAD^+

When D(—)-mandelate dehydrogenase was assayed in the presence of several non-saturating concentrations of the first substrate at a fixed high concentration of the second substrate, the enzymic rate showed a hyperbolic dependence on the substrate concentration, indicating that the enzyme obeys Michaelis-Menten kinetics. Figure 4.10 shows typical primary double-reciprocal plots of the initial velocities of the oxidation reaction against D(—)-mandelate concentration at a series of fixed NAD^+ concentrations. The spread of plots indicates that the reaction catalysed by D(—)-mandelate dehydrogenase proceeds by a sequential mechanism, although product inhibition studies would be required to determine the order in which the cofactor and substrate bind.

The K_m and V values of D(—)-mandelate dehydrogenase with respect to D(—)-mandelate and NAD^+ were determined by the two-substrate method described by Engel (1981). The enzyme was assayed in the presence of several non-saturating concentrations of both substrates. The K_m value for each substrate and the V value were determined when the concentration of the other substrate had been extrapolated to infinity. The initial velocity data were analysed using the Direct Linear method (Eisenthal & Cornish-Bowden, 1974) using the Enzpack computer program (Williams, 1985), from which secondary plots of slopes and intercepts against the reciprocal NAD^+ and D(—)-mandelate concentrations were constructed, enabling the K_m and V values at infinite substrate concentration to be determined. Figure 4.11 shows typical secondary plots of slopes and intercepts against the reciprocal NAD^+ concentration. At pH 9.5, the K_m for

D(—)-mandelate was 319 μM (327, 310) and the K_m for NAD^+ was 71 μM (73, 69). The maximum velocity was 123 units (mg protein)$^{-1}$ (127, 118, 123), which equates to a turnover number (k_{cat}) of 78 s^{-1}.

Although the kinetic data presented here were analysed using the Direct Linear method, they were also analysed and compared with the values obtained with the linear plotting methods (Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee) using the Enzpack computer program. The values obtained for a typical set of initial velocity data with all four methods are given in Table 4.7, and comparison shows that they were generally in good agreement.

4.4.2 Effect of pH on the apparent kinetic coefficients of the oxidation and reduction reactions of D(—)-mandelate dehydrogenase with D(—)-mandelate, NAD^+, phenylglyoxylate and NADH

The apparent K_m and V values were determined by measuring the initial velocities in the presence of several non-saturating concentrations of the first substrate, at a fixed, single high concentration of the second substrate. The apparent kinetic coefficients of D(—)-mandelate dehydrogenase with D(—)-mandelate, NAD^+, phenylglyoxylate and NADH at pH 5.85 (the optimum for the reduction reaction), pH 7.0, pH 8.0 and pH 9.5 (the optimum for the oxidation reaction) are given in Table 4.8. Values for D(—)-mandelate and NAD^+ at pH 5.85 could not be determined because the rate of the oxidation reaction was too low, especially at non-saturating concentrations, to allow accurate estimation of the kinetic coefficients.

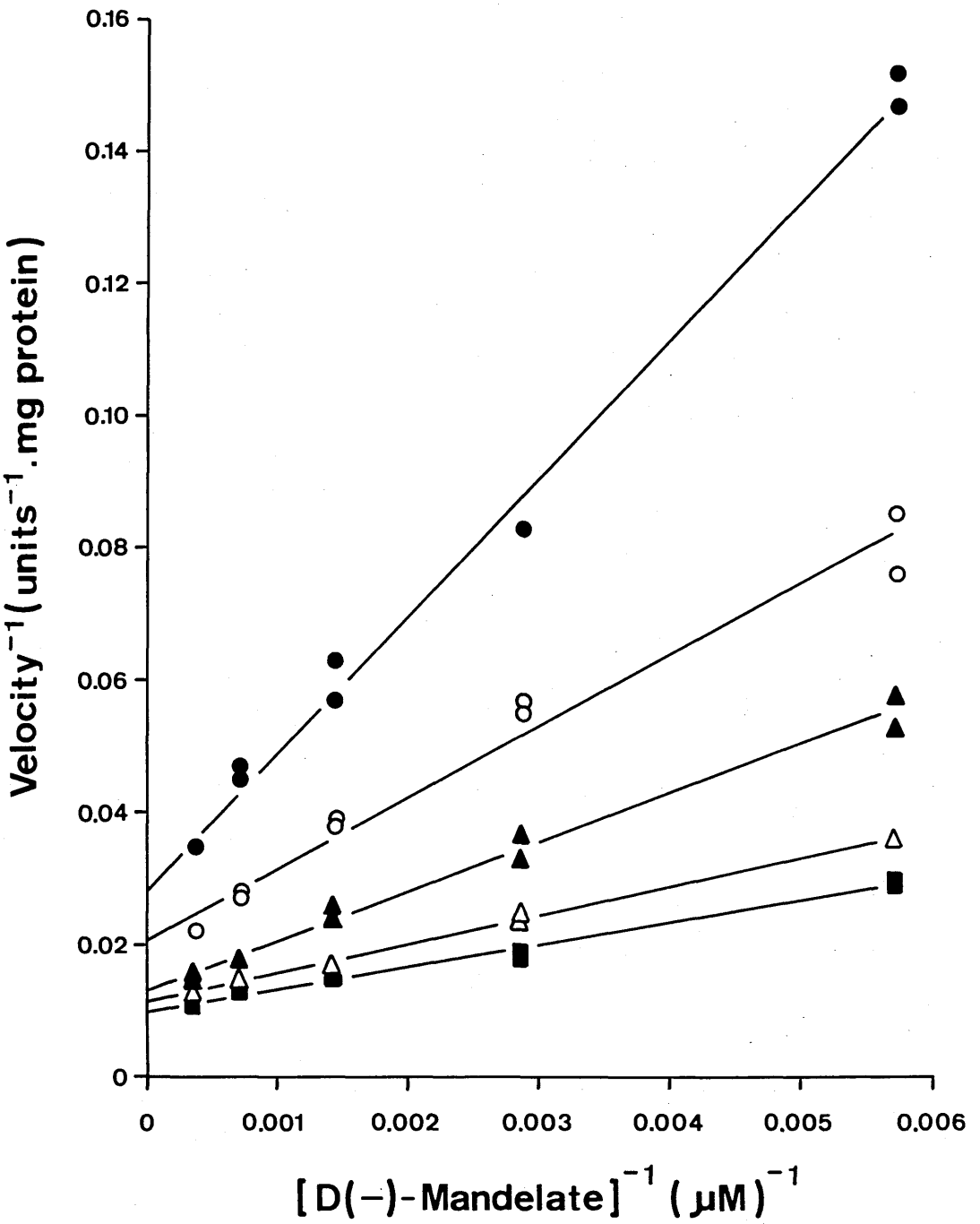


Figure 4.10 Typical plots of initial velocities of the D(—)mandelate dehydrogenase oxidation reaction as a function of the concentrations of D(—)-mandelate and NAD^+

Double-reciprocal plots of the initial velocities against D(—)-mandelate concentration at a series of fixed NAD^+ concentrations : (●), 38 μM; (○), 76 μM; (▲), 153 μM; (△), 307 μM; (■), 615 μM. Enzyme (135 ng) was assayed in 200 mM-Bicine/NaOH buffer (pH 9.5), in duplicate, and where only one point is give the replicates were identical.

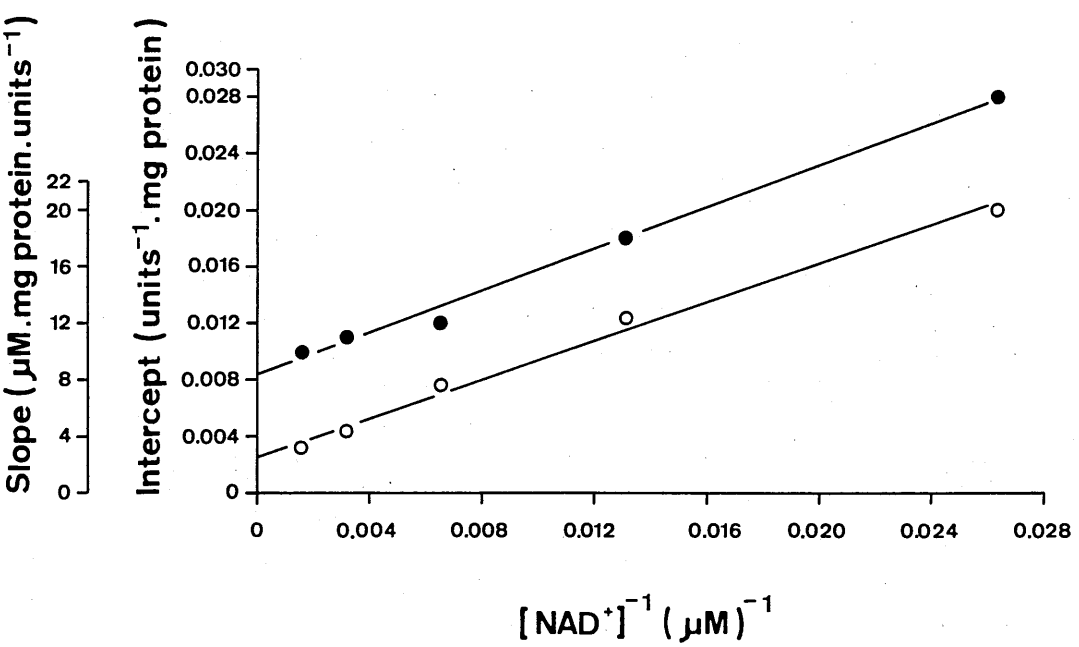


Figure 4.11 Replot of slopes (○) and intercepts (●), derived from the Direct Linear method as a function of the reciprocal NAD^+ concentration

Table 4.7 Comparison of the apparent K_m values (for D(—)-mandelate) and V values obtained when initial velocity data were analysed by the Direct Linear method and the Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plotting methods using the Enzpack computer program

Enzyme was assayed at several D(—)-mandelate concentrations (175 μM, 350 μM, 700 μM, 1400 μM and 2800 μM) at a series of fixed NAD^+ concentrations given in the table.

	D-L		L-B		H-W		E-H	
[NAD^+]	K'_m	V'	K'_m	V'	K'_m	V'	K'_m	V'
38 μM	702	34.9	723	34.8	755	35.3	683	33.8
76 μM	682	55.0	517	47.9	753	57.4	596	51.8
153 μM	591	77.0	549	74.0	597	76.9	557	74.8
307 μM	377	86.8	375	86.3	385	87.1	375	86.5
615 μM	316	97.9	327	99.8	310	97.6	306	97.4

D-L, Direct Linear; L-B, Lineweaver-Burk; H-W, Hanes-Woolf; E-H, Eadie-Hofstee

Table 4.8 Apparent K_m and V values of D(—)-mandelate dehydrogenase with D(—)-mandelate, NAD^+, phenylglyoxylate and NADH at pH 5.85, pH 7.0, pH 8.0 and pH 9.5

The numbers of individual experiments are given in parentheses, except where only two experiments were done in which case both values are given in parentheses. Assays were carried out 200 mM-potassium phosphate/NaOH buffer (pH 5.85; the optimum for the reduction reaction), 200 mM-potassium phosphate/NaOH buffer (pH 7.0), 50 mM-Tris/HCl buffer (pH 8.0) and 200 mM-Bicine/NaOH buffer (pH 9.5; the optimum for the oxidation reaction).

Reaction : D(—)-Mandelate + NAD^+ ————> phenylglyoxylate + NADH + H^+

pH	K'_m D(—)-mandelate (μM)	K'_m NAD^+ (μM)	V' [units (mg protein)$^{-1}$]
5.85	ND	ND	ND
7.0	3050 (3120, 2980)	309 (336, 281)	35.8 ± 5.0 (4)
8.0	372 (380, 364)	72.8 (single expt)	67.4 ± 7.4 (3)
9.5	325 (318, 332)	77.5 (77, 78)	86.7 ± 14.3 (4)

Reaction : Phenylglyoxylate + NADH + H^+ ————> D(—)-mandelate + NAD^+

pH	K'_m phenylglyoxylate (μM)	K'_m NADH (μM)	V' [units (mg protein)$^{-1}$]
5.85	51.5 (41, 62)	38 (37, 39)	572 ± 119.3 (4)
7.0	45.5 (42, 49)	36 (35, 37)	490 ± 109.4 (4)
8.0	43.5 (42.1, 44.9)	14.2 (15.4, 12.9)	311 ± 52.3 (4)
9.5	70.7 (65.4, 76)	26.3 (26.6, 26)	310 ± 41.5 (4)

ND, Not detectable

4.5 Discussion

4.5.1 Quaternary structure, amino acid composition and sequence

D(—)-Mandelate dehydrogenase is a soluble, dimeric enzyme with an estimated M_r of 77 200 (2 x 38 000 subunits) and the detection of a single amino terminal sequence (Section 4.2.3, Table 4.2) indicates that both subunits are identical.

The M_r is similar to that of other NAD^+-dependent, D(—)-enantiomer specific 2-hydroxyacid dehydrogenases. The D(—)-lactate dehydrogenases of lactic acid bacteria and staphylococci are dimers with native M_r values between 70 000 and 80 000 (Garvie, 1980), the D(—)-2-hydroxyisocaproate dehydrogenase of *S. faecalis* is a dimer with a native M_r of 72 000 (Yamazaki & Maeda, 1986) and the D(—)-mandelate dehydrogenase of *L. curvatus* is also a dimer, although it has a somewhat lower M_r of 60 000 (Hummel *et al.*, 1988). The native M_r is also very similar to those of a number of NAD^+-dependent, L(+)-specific 2-hydroxyacid dehydrogenases. Malate dehydrogenase from *E. coli* is a dimer of M_r 65 000 (Fernley *et al.*, 1981), the cytoplasmic and mitochondrial isoenzymes of *S. cerevisiae* are dimers of M_r 75 000 and 68 000 respectively (Hagele *et al.*, 1978), as are their mammalian counterparts e.g. from pig heart, where the enzymes are dimers of M_r 70 000 and 66 000 respectively (Banaszak & Bradshaw, 1975; Birktoft *et al.*, 1982; Birktoft *et al.*, 1987). However, not all malate dehydrogenases are dimeric; the enzyme from *Bacillus subtilis* is a tetramer (M_r 117 000) with a subunit M_r slightly smaller than that of the dimeric forms (Murphey *et al.*, 1967). Likewise, many L(+)-lactate dehydrogenases are tetramers (M_r 140 000), although as with the malate dehydrogenases the subunit M_r values are of the order of 35 000 (Holbrook *et al.*, 1975; Garvie, 1980; Turunen *et al.*, 1987).

In contrast to the soluble, dimeric D(—)-mandelate dehydrogenase of *R. graminis*, the D(—)- and L(+)-mandelate dehydrogenases of *A. calcoaceticus* are monomeric, integral membrane proteins located in the inner cytoplasmic membrane, and have M_r values of 60 000 and 44 000 respectively (Allison *et al.*, 1985*a*,*b*; Hoey *et al.*, 1987). The analogous enzymes of *P. putida* are presumably also monomeric, integral membrane proteins, and although the D(—)-enantiomer specific enzyme has not been purified, the L(+)-mandelate dehydrogenase has recently been purified to near homogeneity and has a M_r , estimated by SDS-polyacrylamide gel electrophoresis, of 44 000 which is in good agreement with the M_r of 43, 352 deduced from the nucleotide sequence (Gunsalus *et al.*, 1953; Stanier *et al.*, 1953; Hegeman *et al.*, 1970; Tsou *et al.*, 1990; C. A. Fewson, personal communication). The L(+)-mandelate dehydrogenase of *R. graminis* also differs from the yeast's D(—)-specific enzyme in that it has a subunit M_r of 60 000 and, although it is readily solubilised during extraction, results from centrifugation experiments suggest that it is membrane "associated". However, the quaternary structure of the enzyme and its intracellular location are as yet unknown (Durham, 1984; M. Yasin, personal communication).

The subunit M_r and quaternary structure of the *R. graminis* D(—)-mandelate dehydrogenase are therefore comparable with those of many other NAD^+-dependent, 2-hydroxyacid dehydrogenases but are quite distinct from those of the $NAD(P)^+$-independent mandelate dehydrogenases.

The amino acid composition revealed a particularly low abundance of cysteine i.e. only one per subunit (Section 4.2.2). This precludes the existence of intra-subunit disulphide bonds, and it is also unlikely that cysteine plays a major role, if any, in inter-subunit contact as the enzyme remains as a dimer in the presence of DTT (Section 4.2.1). Some bacterial lactate dehydrogenases also have a low cysteine content e.g. the enzymes from *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus casei* (Hensel *et al.*, 1977), although most lactate dehydrogenases usually contain between 3 and 10 residues per subunit (Holbrook *et al.*, 1975; Garvie, 1980).

Comparison of nucleotide and amino acid sequences offers the best opportunity to determine whether two or more proteins are related, although comparison of amino acid composition can, in some cases, be used to determine whether a possible relationship exists. The recent cloning and sequencing of the *mdl*A and *mdl*B genes of *P. putida*, which encode mandelate racemase and the membrane-bound L(+)-mandelate dehydrogenase respectively (Ransom *et al.*, 1988; Tsou *et al.*, 1990), enabled their amino acid compositions to be deduced, and comparisons made with the amino acid composition of the *R. graminis* D(—)-mandelate dehydrogenase. The number of residues of each amino acid type, with the exception of tryptophan, were compared and a value for the index of similarity determined according to the method of Cornish-Bowden (1979) which satisfies a formal 92.5% significance test (Cornish-Bowden, 1983). Comparison of the mandelate racemase with the D(—)-mandelate dehydrogenase gave a value of 0.6, and comparison of the two mandelate dehydrogenases resulted in a value of 0.7. Both these values fall between the two threshold values of 0.42, below which relatedness is "strongly" indicated, and 0.93, above which the compositions do not provide grounds for believing a relationship to exist. Therefore, on the basis of amino acid composition data, there is a "weak" indication that the three proteins are related, although for some amino acids D(—)-mandelate dehydrogenase differs significantly from the other two enzymes e.g. in D(—)-mandelate dehydrogenase there are 1 cysteine and 4 methionine residues, whereas in mandelate racemase there are 12 methionines and in L(+)-mandelate dehydrogenase there are 5 cysteine and 11 methionine residues, even though all three proteins are of a comparable size. Furthermore, if there is a "weak" sequence relationship it is likely to occur in stretches of the polypeptides other than their amino termini which show no obvious homology (Section 4.2.3).

The D(—)-mandelate dehydrogenase amino terminal sequence was compared with the sequences in the University of Leeds, OWL composite database (Section 4.2.3). There was no significant homology between any of the archived sequences and that of the amino terminus. Matrix scans, designed to identify particular structural motifs also failed to elucidate any significant homology and, in particular, scanning of the sequence

failed to show the "fingerprint" motif G-X-G-X-X-G...D/E, which is found in the amino terminal sequences of numerous NAD^+-dependent dehydrogenases and, moreover, is known to exist in all known structures of $\beta\alpha\beta$-folds which interact with the ADP moeity of NAD^+ (Wierenga *et al.*, 1986). However, the amino terminal sequence represents only 16% of the entire polypeptide and the complete sequence would be required for a more detailed analysis. Besides, even if little or no sequence homology exists between D(—)-mandelate dehydrogenase and other dehydrogenases this would not preclude the existence of homology at higher structural levels. For example, the main chain of pig heart cytoplasmic and mitochondrial malate dehydrogenases are folded in a very similar manner, resulting in homologous tertiary structures, even though they share only 20% homology at the primary level (Birktoft *et al.*, 1982; Roderick & Banaszak, 1986). D(—)-Mandelate dehydrogenase may well share structural homology with other NAD^+-dependent dehydrogenases but, ultimately, only the determination of the enzyme's three dimensional structure will allow a comparison with other enzymes for which crystal structures are available.

The amino terminal sequence was also analysed to determine whether it contained any runs of amino acids with minimal degeneracy. Such a sequence could then be used in the design of an oligonucleotide "probe" for cloning the D(—)-mandelate dehydrogenase gene. One particularly useful sequence was found, running from tryptophan-19 through to glutamate-27 :

Position	19	20	21	22	23	24	25	26	27
Amino acid	Trp	Ser	Asp	Phe	Gln	Gln	Lys	Phe	Glu
Degeneracy	1	6	2	2	2	2	2	2	2

Although extensive work has been done on yeast genetics, most has been concentrated on a few species, namely *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Rose, 1989), with comparatively little known about the genetic organisation in other genera. There seem to be no reports of genes cloned and sequenced from *R. graminis* and very few from the genus as a whole, notable exceptions being the *leu* gene from *R. glutinis* (Ho & Chang, 1988) and the phenylalanine ammonia lyase gene from *R. rubra* (Filpula *et al.*, 1988; Vaslet *et al.*, 1988). Although there are insufficient data to determine codon utilisation in the genus, it has been noted that for other fungal genes, codons ending in A are used infrequently and, moreover, in the phenylalanine ammonia lyase gene of the related yeast *Rhodosporidium toruloides*, this does not seem to be a consequence of its high GC content (65%), as a similar bias against codons ending in T is not apparent (Orbach *et al.*, 1986; Anson *et al.*, 1987). This observation, along with the fact that *R. graminis* also has a high GC content of 70%

(Storck *et al.*, 1969), which is the highest of all the species in the genus (Fell *et al.*, 1984), may therefore be of assistance when selecting the base sequence for a "probe", especially with respect to the more degenerate third position of each codon.

4.5.2 Cofactors, prosthetic groups and active site metals

D(—)-Mandelate dehydrogenase from *R. graminis* shows the same cofactor specificity as the D(—)-mandelate dehydrogenase from *L. curvatus* and the D(—)-2-hydroxyisocaproate dehydrogenase from *S. faecalis*, with all three enzymes being able to couple the reduction of NAD^+ to the oxidation of D(—)-mandelate and, in the reverse direction, the oxidation of NADH to the reduction of phenylglyoxylate. They are also identical with respect to their inability to use NADP(H) as a cofactor (Yamazaki & Maeda, 1986; Hummel *et al.*, 1988).

The ability of these enzymes to reversibly catalyse the oxidation of mandelate is in contrast to the $NAD(P)^+$-independent D(—)- and L(+)-mandelate dehydrogenases of *A. calcoaceticus*, which essentially catalyse only the oxidation of mandelate. The prosthetic groups of these two bacterial enzymes are flavins, with the D(—)-mandelate dehydrogenase containing FAD and the L(+)-mandelate dehydrogenase containing FMN (Allison *et al.*, 1985*b*; Hoey *et al.*, 1987). The prosthetic group of the *P. putida* L(+)-mandelate dehydrogenase is also likely to be FMN, as the carboxy terminus of this enzyme has extensive sequence homology with the carboxy terminal regions of spinach glycolate oxidase and *S. cerevisiae* cytochrome b_2 lactate dehydrogenase, two enzymes which are known to bind FMN and, moreover, in both these enzymes the carboxy terminal regions are associated with similar $\alpha\beta$ barrel domains that contain the FMN binding site (Lindqvist & Branden, 1985; Xia *et al.*, 1987; Tsou *et al.*, 1990). Likewise, the L(+)-mandelate dehydrogenase of *R. graminis* is $NAD(P)^+$-independent, and is likely to contain flavin as prosthetic group as the purified enzyme is distinctly yellow in colour, is inhibited by mepacrine and is assayed using 2,6-dichloroindophenol as an artificial electron acceptor; a dye routinely used in the assay of the flavin-dependent lactate and mandelate dehydrogenases of *A. calcoaceticus* (Hills & Fewson, 1983*a*; Allison *et al.*, 1985*a*,*b*; Hoey *et al.*, 1987; M. Yasin, personal communication).

Therefore, on the basis of cofactor specificity there are at least two clearly definable groups of mandelate dehydrogenase; pyridine nucleotide-dependent enzymes and flavin-dependent enzymes, which differ with respect to their modes of electron transport.

Both *A. calcoaceticus* and *R. graminis* can grow on D(—)- or L(+)-mandelate as the sole source of carbon and energy (Kennedy & Fewson, 1968*a*; Hills & Fewson, 1983*a*; Durham *et al.*, 1984) and, as chemoheterotrophes, they must be able to derive energy (in the form of ATP) from the breakdown of these aromatic substrates. Although the oxidation of mandelate is only one of the steps in the pathway yielding reducing equivalents, it is an energetically important reaction because oxidation of the reduced cofactor/prosthetic group can be coupled to electron transport and oxidative phosphorylation. This has been confirmed experimentally with *A. calcoaceticus* wild type strain NCIB 8250, which gave a

higher maximum molar growth yield (in continuous culture) when grown on L(+)-mandelate [70.6 g dry wt. (mol substrate)$^{-1}$] than it did when grown on phenylglyoxylate [66.5 g dry wt. (mol substrate)$^{-1}$] (Fewson, 1985). However, a comparison of growth yield on D(—)-mandelate could not be made as this strain is unable to utilise this enantiomer (Kennedy & Fewson, 1968*b*), although a similar higher growth yield would presumably be obtained for strains that can grow on this substrate e.g. wild type strain EBF 65/65 (Hills & Fewson, 1983*a*). Unlike the prosthetic groups of the *A. calcoaceticus* mandelate dehydrogenases, the NADH formed by the *R. graminis* D(—)-mandelate dehydrogenase can dissociate from the enzyme, whereupon it presumably feed electrons to the FMN prosthetic group of NADH dehydrogenase in the inner mitochondrial membrane. Electron transport along the respiratory chain, to oxygen as the terminal acceptor, probably yields 3 moles of ATP per mole of D(—)-mandelate oxidised. However, the *A. calcoaceticus* mandelate dehydrogenases feed electrons from the substrate to the enzyme-bound flavin, which presumably forms part of an electron transport system in the inner cytoplasmic membrane with cytochromes *o* or a_2 (*d*) as terminal oxidases and O_2 as the terminal electron acceptor (Hardy & Dawes, 1985; Fewson, 1988*b*). As the bacterial enzymes feed electrons into the respiratory chain at the level of reduced flavin the overall maximum net production of ATP is therefore probably lower, at only 2 ATP per mole of mandelate oxidised. Although growth yield experiments like those carried out with *A. calcoaceticus* have not been carried out with *R. graminis*, the validity of the above argument could be tested by growing the yeast in continuous culture on either D(—)-mandelate or L(+)-mandelate. As the organism has an NAD^+-dependent D(—)-mandelate dehydrogenase and a flavin-dependent L(+)-mandelate dehydrogenase, the growth yield on each enantiomer could be compared to determine whether NAD^+-dependent oxidation of mandelate is more productive in terms of ATP yield than is flavin-dependent oxidation.

As *R. graminis* D(—)-mandelate dehydrogenase is an NAD(H)-dependent enzyme, it can be classified according to which of the two hydrogens at the C_4 position of the dihydronicotinamide ring of NADH it transfers to the substrate (phenylglyoxylate) during the reduction reaction. The two hydrogens attached at the C_4 position of the ring are termed pro-*R* (A hydrogen) and pro-*S* (B hydrogen), and lie on the *re* and *si* faces of the ring respectively; the two faces of the dihydronicotinamide ring being labelled according to the *RS* convention as the carbon at position 4 is prochiral (Fersht, 1985).

The two hydrogens, apart from being sterically different are also enzymically non-equivalent with individual dehydrogenases transferring only one of them. Approximately 200 dehydrogenases have so far been examined, of which about half transfer the pro-*R* hydrogen with the other half transferring the pro-*S* hydrogen (Benner *et al.*, 1987, 1989). Crystallographic studies have shown that in the case of enzymes that transfer the pro-*R* hydrogen, e.g. dogfish lactate dehydrogenase, porcine cytoplasmic malate dehydrogenase and horse liver alcohol dehydrogenase, the dihydronicotinamide ring is orientated in an *anti* conformation with respect to the

glycosidic bond, whereas an enzyme like lobster glyceraldehyde-3-phosphate dehydrogenase, which transfers the pro-*S* hydrogen has the ring orientated in the *syn* conformation (Rossmann *et al.*, 1975; You *et al.*, 1978). It is therefore likely that *R. graminis* D(—)-mandelate dehydrogenase, which transfers the pro-*R* hydrogen [Section 4.3.5 (b)], binds NADH in the *anti* conformation, thereby orientating the *re* face of the dihydronicotinamide ring and pro-*R* hydrogen towards the bound substrate. It is also likely, according to Bentley (1970), that the D(—)-mandelate dehydrogenase of *L. curvatus* and the D(—)-2-hydroxyisocaproate dehydrogenase of *S. faecalis* [which has marked D(—)-mandelate dehydrogenase activity (Yamazaki & Maeda, 1986)] should also transfer the pro-*R* hydrogen because he concluded that "the stereospecificity of a particular enzyme does not depend on the source of the enzyme". It would be of significant interest to determine the stereospecificity of hydride transfer of these two other enzymes, to determine whether Bentley's generalisation is true for NAD(H)-dependent mandelate dehydrogenases.

An example that clearly does fit this generalisation is that of malate dehydrogenase, as the enzymes from a diverse range of organisms including archaebacteria (*Sulfobolus acidocaldarius*, *Thermoplasma acidophilum* and *Halobacter halobium*), eubacteria (*Bacillus subtilis* and *Pseudomonas putida*), fungi (*Neurospora crassa*), plants (potato and wheat germ), arthropods (*Drosophila melanogaster*), birds (chicken) and mammals (pig) all transfer the pro-*R* hydrogen (Loewus *et al.*, 1955; Graves *et al.*, 1956; Davies *et al.*, 1972; Do Nascimento & Davies, 1975; Arnold *et al.*, 1976; Bernstein & Everse, 1978; Goerisch *et al.*, 1985; Allemann *et al.*, 1988). Likewise, lactate dehydrogenases from an equally diverse range of organisms all transfer the pro-*R* hydrogen (You, 1982 and references therein). Although Bentley's rule holds true for the majority of comparisons, not all enzymes catalysing the same reaction, but which come from different sources, share the same stereospecificity with respect to hydride transfer. For example, alcohol dehydrogenases from *S. cerevisiae* and horse liver transfer the pro-*R* hydrogen, whereas the enzyme from *Drosophila melanogaster* transfers the pro-*S* hydrogen (Fisher *et al.*, 1953; Vennesland, 1956; Levy & Vennesland, 1957; Benner *et al.*, 1985). Examples such as alcohol dehydrogenases which show heterogeneity with respect to hydride transfer contradict historical models that assume that stereospecificity and substrate specificity in dehydrogenases are highly conserved traits, and that enzymes from all organisms that act on the same substrate are homologous. Such discrepancies led Benner (1982) to propose an alternative functional model that correlates stereospecificity with the redox potential (or thermodynamic stability) of the substrate, and more recently Benner *et al.* (1985) updated this model to include three hypotheses which state : (1) which hydrogen is transferred is controlled by the conformation of the bound NADH, (2) different confomers of NADH have different redox potentials, and therefore (3) functionally optimal enzymes have evolved to match the redox potential of bound NADH to the redox potential of the natural substrate. These models assume that dehydrogenases which reduce

unstable carbonyls evolved to transfer the pro-*R* hydrogen but those which reduce stable carbonyls evolved to transfer the pro-*S* hydrogen, but neither stereospecificity is strongly selected in those enzymes which reduce carbonyl substrates with "intermediary stability" i.e. those with an equilibrium constant of approximately 10^{-11} M (Allemann *et al.*, 1988). Therefore, with alcohol dehydrogenases which have an equilibrium constant close to this value ($K'_{eq} = 8 \times 10^{-12}$ M, Allemann *et al.*,1988), neither stereospecificity is strongly selected and this has resulted in both pro-*R* and pro-*S* enzymes. Examination of a number of enzymes (Table 4.9) shows that an equilibrium constant of 10^{-11} M falls at the "break" in correlation between the equilibrium constant and stereochemistry (Benner *et al.*, 1989). Interestingly, *R. graminis* D(—)-mandelate dehydrogenase has an equilibrium constant of 1.59×10^{-11} M (Section 4.3.4) which also falls at this "break" in the correlation, and this might indicate that the stereospecificity of hydride transfer has not been strongly selected during the evolution of the NAD(H)-dependent mandelate dehydrogenases, as apparently seems the case with alcohol dehydrogenases or, alternatively, transfer of the pro-*R* hydrogen may reflect a functional advantage in the reaction catalysed by the ancestral enzyme of D(—)-mandelate dehydrogenase, and although the modern enzyme has retained pro-*R* specificity, it might not necessarily be optimal for the interconversion of D(—)-mandelate and phenylglyoxylate.

Although some bacterial and fungal 2-hydroxyacid dehydrogenases have been shown to require divalent metal ions for activity e.g. the Zn^{2+}-dependent D(—)-lactate dehydrogenase of *Megasphaera elsdenii* and the Zn^{2+}-dependent D(—)-lactate : cytochrome c oxidoreductase of *S. cerevisiae* (Hatefi & Stiggall, 1976; Olson & Massey, 1979), the activity of *R. graminis* D(—)-mandelate dehydrogenase was not effected by a range of salts, metal ions or metal chelating agents (Section 4.3.7) suggesting that, like the mandelate dehydrogenases of *A. calcoaceticus*, the D(—)-mandelate dehydrogenase of *L. curvatus* and the D(—)-2-hydroxyisocaproate dehydrogenase of *S. faecalis*, its activity is independent of metals or other ions (Allison *et al.*, 1985*b*; Yamazaki & Maeda, 1986; Hoey *et al.*, 1987; Hummel *et al.*, 1988). However, although sensitivity to metal chelating agents is a general indicator of metal dependence, insensitivity does not prove that the enzyme does not have bound metal, as the chelators may be sterically prevented from removing the metal ion.

Table 4.9 Stereospecificity of dehydrogenases arranged by the p K_{eq} values for their physiological reaction

Information is taken from this thesis and Benner *et al.* (1989).

Name	pK_{eq}	Stereochemistry
Glyoxylate reductase	17.5	pro-*R*
Glyoxylate reductase ($NADP^+$)	17.5	pro-*R*
Tartronate semialdehyde reductase	13.3	pro-*R*
Glycerate dehydrogenase	13.3	pro-*R*
Glycerol dehydrogenase ($NADP^+$)	12.8	pro-*R*
Hydroxypyruvate reductase	12.4	pro-*R*
Malate dehydrogenase ($NADP^+$)	12.1	pro-*R*
Malate dehydrogenase	12.1	pro-*R*
Malic enzyme	12.1	pro-*R*
Malic enzyme ($NADP^+$)	12.1	pro-*R*
L(+)-Lactate dehydrogenase	11.6	pro-*R*
D(—)-Lactate dehydrogenase	11.6	pro-*R*
Alcohol dehydrogenase (*S. cerevisiae*)	11.4	pro-*R*
Alcohol dehydrogenase (*Drosophila*)	11.4	pro-*S*
Glycerol-2-dehydrogenase	11.3	pro-*R*
Glycerol-3-phosphate dehydrogenase	11.1	pro-*S*
Homoserine dehydrogenase	10.9	pro-*R*
Carnitine dehydrogenase	10.9	pro-*S*
D(—)-Mandelate dehydrogenase	**10.8**	**pro-*R***
3-Hydroxyacyl CoA dehydrogenase	10.5	pro-*S*
3-Hydroxybutyrate dehydrogenase	8.9	pro-*S*
3-Hydroxysteroid dehydrogenase	8.0	pro-*S*
Estradiol 17-β-dehydrogenase	7.7	pro-*S*
Testosterone β-dehydrogenase	7.6	pro-*S*
3-Oxoacyl ACP reductase	7.6	pro-*S*
β-Hydroxysteroid dehydrogenase	7.6	pro-*S*

4.5.3 Substrate and inhibitor specificity

R. graminis D(—)-mandelate dehydrogenase is completely stereospecific for its substrate, a property shared with all other purified mandelate dehydrogenases (Allison *et al.*, 1985*b*; Yamazaki & Maeda, 1986; Hoey *et al.*, 1987; Hummel *et al.*, 1988), and although L(+)-mandelate cannot be oxidised by the enzyme, it is a competitive inhibitor and is therefore able to bind at the active site. Similarly, *A. calcoaceticus* D(—)-mandelate dehydrogenase activity is inhibited by L(+)-mandelate (80% inhibition in the presence of an equimolar concentration of the two enantiomers) as is the L(+)-mandelate dehydrogenase activity by D(—)-mandelate, although it is a relatively weaker inhibitor (Allison *et al.*, 1985*b*; Hoey *et al.*, 1987). L(+)-Mandelate appears also to be an inhibitor of the D(—)-2-hydroxyisocaproate dehydrogenase of *S. faecalis* as the enzymic activity in the presence of 10 mM-D,L-mandelate is only 93% of the activity with 5 mM-D(—)-mandelate (Yamazaki & Maeda, 1986).

R. graminis D(—)-mandelate dehydrogenase was active with a number of ring-substituted mandelates and could tolerate substitutions at the 2, 3, 4 and 3,4 positions of the phenyl ring (Table 4.4). Unfortunately, determination of the appropriate specificity constants (k_{cat} /apparent K_m) and subsequent comparison of the various substrates could not made for the reasons outlined in Section 4.3.6. The only other compounds which the enzyme could use as substrates [apart from D(—)-mandelate and phenylglyoxylate] were thiopheneglyoxylate and phenylpyruvate. Phenyllactate, the 2-hydroxy form of phenylpyruvate was found not to be a substrate but did act as an inhibitor (Table 4.5). This apparent discrepancy in being able to assay the enzyme for phenylpyruvate reduction but not for phenyllactate oxidation can be explained when one considers the difference in velocity between phenylglyoxylate reduction and D(—)-mandelate oxidation at their respective pH optima. The maximum velocity of phenylglyoxylate reduction is 6.6 times greater than D(—)-mandelate oxidation [572 units (mg protein)$^{-1}$ against 86.7 units (mg protein)$^{-1}$, Section 4.4.2], and if the same ratio exists with the phenylpyruvate/phenyllactate pair then the observed change in A_{340} when assaying for the oxidation of phenyllactate (reduction of NAD^+) would barely be detectable as the rate with phenylpyruvate was itself low.

Comparison of the compounds that are substrates show them to contain some form of ring structure (an aromatic ring, a non-aromatic six carbon ring or a thiophene ring), and it is possible that binding of the ring plays an important role in orientating the substrate at the active site. Correct binding of the ring may also confer specificity to these compounds, by contributing to the binding energy and therefore to the energy required for catalysis. In the active site of mandelate racemase the aromatic side chains of tyrosine and phenylalanine residues solvate the aromatic ring of mandelate (G. Petsko, personal communication), and similar specific interactions in the D(—)-mandelate dehydrogenase active site may account for the enzyme's specificity for ring-containing substrates. The possibility of a specific interaction between the active site residues and the hydrophobic ring seems more likely when one considers that a range of

aliphatic 2-hydroxy and 2-oxocarboxylic acids are not substrates but are inhibitors of enzymic activity (Tables 4.5 & 4.6) i.e. although they bear hydrophobic side chains and can bind at the active site, they lack a ring structure and are therefore incorrectly orientated for catalysis. Certain-ring containing compounds were also substrates of the *S. faecalis* D(—)-2-hydroxyisocaproate dehydrogenase and the *L. curvatus* D(—)-mandelate dehydrogenase. In addition to D(—)-mandelate and phenylglyoxylate, the *S. faecalis* enzyme can also oxidise D(—)-phenyllactate (61% of the mandelate rate) and reduce phenylpyruvate (94% of the phenylglyoxylate rate) and, likewise, the *L. curvatus* enzyme uses phenyllactate (22% of the mandelate rate) and phenylpyruvate (63% of the phenylglyoxylate rate). However, both enzymes show poor, if any, activity with ring-hydroxylated analogues; the *S. faecalis* enzyme being unable to use 4-hydroxyphenylglyoxylate even though it shows marked activity with phenylglyoxylate, and the activity of the *L. curvatus* enzyme drops from 63% of the mandelate rate with phenylpyruvate to only 4% with 4-hydroxyphenylpyruvate (Yamazaki & Maeda, 1986; Hummel *et al.*, 1988). This indicates that the bacterial enzymes, unlike the *R. graminis* D(—)-mandelate dehydrogenase, do not readily tolerate ring-hydroxylation.

Both the *L. curvatus* D(—)-mandelate dehydrogenase and the *S. faecalis* D(—)-2-hydroxyisocaproate dehydrogenase also differ from the *R. graminis* D(—)-mandelate dehydrogenase, in that many of the aliphatic 2-hydroxy and 2-oxocarboxylic acids which inhibit activity of the yeast enzyme are good substrates of the bacterial enzymes (Yamazaki & Maeda, 1986; Hummel *et al.*, 1988). In order to compare the relative specificities of the bacterial enzymes for each of the substrates, the kinetic data presented by the authors were used to determine the specificity constant (k_{cat}/K'_m) (Table 4.10). Of the 13 substrates of the *L. curvatus* D(—)-mandelate dehydrogenase, the highest specificity constant was obtained with 2-oxo-3-methylvalerate, with one straight-chain and three other branched-chain 2-oxocarboxylic acids all giving values significantly higher than phenylglyoxylate. Of the 10 substrates of the *S. faecalis* D(—)-2-hydroxyisocaproate dehydrogenase, the highest value was obtained with 2-oxoisocaproate which also gave the highest relative velocity (Yamazaki & Maeda, 1986), and like the *Lactobacillus* enzyme other branched-chain acids gave higher values than phenylglyoxylate e.g. 2-oxoisovalerate and 2-oxo-3-methylvalerate. It is usually considered that the higher the specificity constant the closer the structure of the substrate transition-state intermediate is to the enzyme's active site (Fersht, 1985), and therefore it is probable that the substrates of these enzymes *in vivo* are branched 2-oxocarboxylic acids such as 2-oxoisovalerate, 2-oxoisocaproate and 2-oxo-3-methyl valerate which are formed by the oxidative deamination of valine, leucine and isoleucine respectively. Moreover, there is no evidence to suggest that *L. curvatus*, *S. faecalis* or a number of other bacteria from the genera *Lactobacillus* and *Leuconostoc* which show marked NAD^+-dependent mandelate dehydrogenase activity are capable of growing on D(—)-mandelate as the sole source of carbon and energy (Yamazaki & Maeda, 1986; Hummel *et al.*, 1988; Smith, 1989). These organisms therefore seem to have a fortuitous

ability to oxidise D(—)-mandelate/reduce phenylglyoxylate endowed by the broad specificity of these enzymes, which unlike the *R. graminis* enzyme do not form part of a metabolic pathway for the catabolism of D(—)-mandelate.

A. calcoaceticus, on the other hand, can grow not only on D(—)- or L(+)-mandelate as the sole source of carbon and energy but also on a number of ring-substituted mandelates. The wild type strain NCIB 8250 can grow on D,L-2-hydroxymandelate, D,L-4-hydroxymandelate, D,L-3,4-dihydroxymandelate and D,L-4-hydroxy-3-methoxymandelate. Other ring-substituted mandelates do not support growth but can be partially metabolised e.g. D,L-4-bromomandelate, D,L-4-chloromandelate, D,L-3-hydroxymandelate and D,L-3-hydroxy-4-methoxymandelate are degraded to the level of their corresponding ring-substituted benzoate, whereas D,L-4-fluoromandelate is oxidised beyond the benzoate level (Kennedy & Fewson, 1968*b*). Although the substrate specificity of the purified mandelate dehydrogenases of *A. calcoaceticus* with respect to these compounds has not been tested, the ability of this bacterium to grow on or partially metabolise these compounds is presumably due to substrate ambiguity of the mandelate dehydrogenases and other enzymes of the mandelate pathway (Fewson, 1988*b*). The only substrates with which the purified enzymes from *A. calcoaceticus* have been tested are D(—)- and L(+)-lactate and these were of particular interest because of the striking similarities between the $NADP^+$-independent mandelate and lactate dehydrogenases in this organism (Hoey *et al.*, 1987). The L(+)-mandelate dehydrogenase cannot use L(+)-lactate as a substrate but the D(—)-mandelate dehydrogenase can use D(—)-lactate, albeit with an apparent K_m 12.5 times higher and a maximum velocity 5 times lower than with D(—)-mandelate (Allison *et al.*, 1985*b*; Hoey *et al.*, 1987).

The inability of a number of potential metabolic inhibitors to affect the D(—)-mandelate dehydrogenase of *R. graminis* (Section 4.3.6) is similar to other NAD^+-dependent dehydrogenases of the mandelate pathway e.g. benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *A. calcoaceticus* (MacKintosh & Fewson, 1988*b*), and this supports the view that feedback inhibition is not an important mechanism in the regulation of peripheral metabolic pathways (Fewson, 1988*b*). In contrast, NAD^+-dependent dehydrogenases of central metabolic pathways often show regulation of activity by small effector molecules e.g. lactate dehydrogenase of *Bacillus stearothermophilus* is activated by fructose 1,6-bisphosphate, which builds up when glucose is plentiful and thereby increases flux to lactate, and the D(—)-lactate dehydrogenase of the fungus *Pythium debaromyces* is allosterically inhibited by GTP thereby diverting the flux of pyruvate away from D(—)-lactate towards oxaloacetate during gluconeogenesis (Clarke *et al.*, 1989*b*; Le John, 1971).

Table 4.10 Substrate specificity of the *L. curvatus* D(—)-mandelate dehydrogenase and the *S. faecalis* D(—)-2-hydroxyisocaproate dehydrogenase.

Information is taken from Yamazaki & Maeda (1986) and Hummel *et al.* (1988). The values of k_{cat} were determined from the maximum velocities of 2122 units (mg protein)$^{-1}$ and 897 units (mg protein)$^{-1}$, and subunit M_r values of 30 000 and 34 000 for the *L. curvatus* and *S. faecalis* enzymes respectively.

L. curvatus D(—)-mandelate dehydrogenase

Compound	*V* (relative %)	k_{cat} (s^{-1})	K'_m (mM)	k_{cat}/K'_m (M^{-1} s^{-1})
Phenylglyoxylate	100	1060	0.22	4.8×10^6
2-Oxobutyrate	51	541	0.55	9.8×10^5
2-Oxoxvalerate	76	806	0.17	4.7×10^6
2-Oxocaproate	74	784	0.10	7.8×10^6
2-Oxooctanoate	6	64	0.35	1.8×10^5
2-Oxo-3-methylbutyrate	176	1866	0.18	1.0×10^7
2-Oxo-3-methylvalerate	119	1261	0.095	1.3×10^7
2-Oxoisocaproate	76	806	0.090	9.0×10^6
2-Oxo-4-methyl-mercaptobutyrate	68	721	0.11	6.6×10^6
Trimethylpyruvate	15	159	5.4	2.9×10^4
Phenylpyruvate	63	668	0.15	4.5×10^6
4-Hydroxyphenylpyruvate	4	42	0.65	6.5×10^4
3-(3,4)-Dihydrophenylpyruvate	4	42	0.37	1.1×10^5

S. faecalis D(—)-2-hydroxyisocaproate dehydrogenase

Compound	*V* (relative %)	k_{cat} (s^{-1})	K'_m (mM)	k_{cat}/K'_m (M^{-1} s^{-1})
Phenylglyoxylate	100	510	3.2	1.6×10^5
2-Oxobutyrate	4	20	7.7	2.6×10^3
2-Oxovalerate	22	112	3.3	3.4×10^4
2-Oxocaproate	29	148	2.3	6.4×10^4
2-Oxoisovalerate	53	270	0.4	6.8×10^5
2-Oxoisocaproate	121	617	0.7	8.8×10^5
2-Oxo-3-methylvalerate	63	321	0.5	6.4×10^5
Phenylpyruvate	94	479	7.0	6.8×10^4
Chloropyruvate	66	337	33.0	1.0×10^4
Bromopyruvate	82	418	7.3	5.7×10^4

4.5.4. Kinetics

R. graminis D(—)-mandelate dehydrogenase is similar to both the *L. curvatus* D(—)-mandelate dehydrogenase and the *S. faecalis* D(—)-2-hydroxyisocaproate dehydrogenase, but not the *R. graminis* L(+)-mandelate dehydrogenase or *A. calcoaceticus* mandelate dehydrogenases with respect to the pH values at which they show optimal activity. The three NAD^+-dependent enzymes catalyse a reversible reaction and show two pH optima, with a higher pH optimum for the oxidation reaction than the reduction reaction. The optimum pH for the oxidation reaction of the *R. graminis*, *L. curvatus* and *S. faecalis* enzymes are 9.5, 8.5 and 9.2 respectively, whereas for the reduction reaction they are 5.85, 6.0 and 4.5 respectively (Figure 4.5; Yamazaki & Maeda, 1986; Hummel *et al.*, 1988). These results are also very similar to a number of other bacterial and fungal NAD^+-dependent, 2-hydroxyacid dehydrogenases where the oxidation of the 2-hydroxyacid shows a higher pH optimum than the reduction of the corresponding 2-oxoacid (Le John, 1971; Garvie, 1980). By contrast, each of the $NAD(P)^+$-independent mandelate dehydrogenases which catalyse an essentially irreversible reaction show a single pH optimum, and these are similar to each other with values of 7.5, 8.0 and 7.5 for the *R. graminis* L(+)-mandelate dehydrogenase and *A. calcoaceticus* D(—)- and L(+)-mandelate dehydrogenases respectively (Allison *et al.*, 1985*b*; Hoey *et al.*, 1987; M. Yasin, personal communication).

The pH was also shown to have a marked effect on the apparent K_m values for the substrates and cofactors of the yeast enzyme, especially those for D(—)-mandelate and NAD^+ (Table 4.8). Although there was relatively little change in the apparent K_m values for phenylglyoxylate or NADH, a change from pH 9.5 to neutrality resulted in an almost 10-fold increase in the apparent K_m value for D(—)-mandelate and a 4-fold increase for NAD^+. Furthermore, at all of the pH values tested, the apparent K_m values for phenylglyoxylate and NADH were significantly lower than for D(—)-mandelate and NAD^+, and the apparent maximum velocities were significantly greater in the reverse (reduction) reaction than in the forward (oxidation) reaction (Section 4.4.2). Therefore, even at pH 9.5 (the optimum for the forward reaction), the enzyme is a better phenylglyoxylate reductase than it is a D(—)-mandelate dehydrogenase. However, D(—)-mandelate dehydrogenase is induced during the growth of *R. graminis* on mandelate (Durham *et al.*, 1984) and presumably the reaction is driven in the physiological forward direction by the decarboxylation of the product by phenylglyoxylate decarboxylase and by the oxidation of the NADH formed.

4.5.5 Active site thiols

One property in which the *R. graminis* D(—)-mandelate dehydrogenase clearly differs from all other purified mandelate dehydrogenases is its marked insensitivity to a range of thiol reagents (Section 4.3.8), which suggests that the single cysteine residue identified by amino acid analysis (Section 4.2.2) is not essential for catalysis. Alternatively, this residue might be involved in catalysis but is inaccessible to these reagents. However, this seems unlikely as 4-(chloromercuri)benzoate, a reagent not too dissimilar in structure to mandelate, should be able to gain access to the active site, especially as several aromatic and aliphatic mandelate analogues can do so.

The insensitivity of the yeast enzyme is in stark contrast to the mandelate dehydrogenases of *A. calcoaceticus*, which are susceptible to these reagents; in particular the D(—)-mandelate dehydrogenase is strongly inhibited by 4-(chloromercuri)benzoate; less than 1 μM producing 50% inhibition after only a 10 min incubation at 27°C (Allison *et al.*, 1985*b*; Hoey *et al.*, 1987). The *R. graminis* enzyme also differs from the *S. faecalis* D(—)-2-hydroxyisocaproate dehydrogenase [complete inhibition at 0.5 mM-4-(chloromercuri)benzoate], the *L. curvatus* D(—)-mandelate dehydrogenase [94% inhibition at 100 μM-4-(chloromercuri)benzoate] and many lactate dehydrogenases which are inhibited by thiol reagents (Holbrook *et al.*, 1975; Yamazaki & Maeda, 1986; Hummel *et al.*, 1988). These results suggest that the bacterial mandelate dehydrogenases may have active site cysteine residue(s), although sensitivity to thiol reagents does not prove the involvement of cysteine side chain(s) in the catalytic mechanism (especially in the absence of substrate protection and stoicheiometry studies) and can neither differentiate between a catalytically essential residue and a structurally essential residue, modification of which leads to inactivation due to steric or other effects.

CHAPTER 5

AFFINITY LABELLING OF D(—)-MANDELATE DEHYDROGENASE WITH D(—)-2-(BROMOACETYL)MANDELATE

5.1 Introduction

In the absence of X-ray crystallographic data, chemical modification offers an opportunity to investigate the active site of an enzyme, and may yield information as to the topology of the active site as well as the role of specific amino acid side chains in substrate binding and catalysis (Plapp, 1982; Fersht, 1985). However, chemical modification is not restricted to the study of the active site and has proved useful in the investigation of other aspects of protein structure and function e.g. allosteric and cooperative properties, alteration of enzyme specificity and determination of an enzyme's quaternary structure (Eyzaguirre, 1987). Numerous chemicals are available to modify proteins and they may be divided into two broad categories; the group-specific reagents and the affinity labels although, in some cases, a group-specific reagent may also act as an affinity label. Although many of the former group are selective towards the amino acids with which they react few, if any, are absolutely specific (Eyzaguirre, 1987). For example the reagent *N*-ethylmaleimide reacts primarily with the thiolate anion of the cysteine side chain, but will also react with amino groups, and likewise *N*-bromosuccinamide which reacts primarily with tryptophan will also react with tyrosine (Means & Feeney, 1971). Furthermore, even if a reagent is group-specific it may react at more than one site on a protein, making identification of the active site residue(s) difficult.

An alternative method is to use an affinity label which is a chemically reactive compound analogous to the natural substrate. The enzyme binds the label in a manner similar to that of the substrate and, therefore, brings a reactive group into close proximity with the amino acid side chains at or near the active site. Once bound, the label may dissociate from the enzyme or it may react with an active site residue, leading to covalent bond formation and enzyme inactivation. The inactivation of an enzyme by an affinity label is described by the following equation (Meloche, 1967) :

$$E + I \longleftrightarrow E.I \longrightarrow E\text{-}X$$

where E is the enzyme, I is the inhibitor, E.I is the reversible enzyme.inhibitor complex and E-X is the inactive, covalently modified enzyme. The dissociable complex formed between the enzyme and inhibitor prior to covalent modification is analogous to the enzyme.substrate complex formed during catalysis. If a compound is to be considered as an affinity label it must satisfy four important criteria (Meloche, 1967) :

(1) It should inhibit the enzyme completely and irreversibly.

(2) The inactivation should show rate saturation i.e. there is a maximum finite of inactivation.

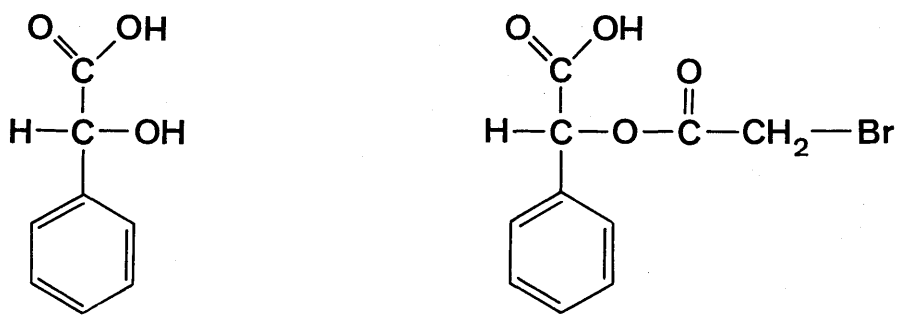
(3) The inactivation should be protected by the substrate or by reversible inhibitors of the enzyme i.e. if the affinity label is binding at the active site, the presence of competing substrate or reversible inhibitor will reduce the rate of inactivation.

(4) The binding of the inhibitor should be stoicheiometric i.e. one inhibitor molecule should bind per active site.

D(—)-Mandelate dehydrogenase catalyses the reversible interconversion of D(—)-mandelate and phenylglyoxylate, removing two hydrogens from D(—)-mandelate in the oxidation reaction and transferring two hydrogens to phenylglyoxylate in the reduction reaction (Section 4.3.1). The overall reaction is analogous to that of the NAD^+-dependent lactate dehydrogenases which catalyse the reversible interconversion of lactate and pyruvate (Holbrook *et al.*, 1975). In the oxidation of lactate by lactate dehydrogenase, the two hydrogens are removed from lactate in the form of a hydride ion from the central chiral carbon and a proton from the hydroxyl group; the hydride ion being transferred to the C_4 position of the dihydronicotinamide ring of NAD^+, and the proton abstracted by the unprotonated imidazole group of an active site histidine (Clarke *et al.*, 1989*a*). Since D(—)-mandelate dehydrogenase is also an NAD^+-dependent enzyme [Section 4.3.5 (a)] and part of the overall reaction involves hydride transfer [Section 4.3.5 (b)], an attempt was made to identify the active site residue which might be involved in removing the proton from the mandelate hydroxyl group during the oxidation reaction.

In order to probe the active site for reactive and accessible nucleophilic groups which might mediate this function, the compound D(—)-2-(bromoacetyl)mandelic acid was chosen as a potential affinity label as it retains both the phenyl and carboxylate groups found in the natural substrate, but also includes a reactive and electrophilic bromoacetyl group (Figure 5.1). The bromoacetyl group was incorporated at the position normally occupied by the hydroxy/carbonyl groups of D(—)-mandelate/phenylglyoxylate respectively. If the phenyl and carboxylate groups of D(—)-2-(bromoacetyl)mandelate bind to the enzyme in the same positions as the corresponding groups of D(—)-mandelate, the bromoacetyl function should be positioned to intercept the nucleophilic amino acid side chain involved in proton transfer.

In the course of the present work, methods have been developed for the synthesis and purification of D(—)-2-(bromoacetyl)mandelic acid, and its physical and chemical characteristics were determined to confirm the synthesis of the compound. Although the synthesis of this compound had been reported using a different chemical route, no attempt had previously been made to characterize it (Tacwor, 1956). Furthermore, it had been synthesized as a general bacteriocide/fungicide and not as an inhibitor of any specific enzyme. The inactivation of D(—)-mandelate dehydrogenase by the label is reported, along with the procedures used to isolate and characterize the labelled active site peptides. Attempts were also made identify the modified amino acid.



D(—)-Mandelic acid

D(—)-2-(Bromoacetyl)mandelic acid

Figure 5.1 Structure of D(—)-mandelic acid and D(—)-2-(bromoacetyl)mandelic acid

5.2 Synthesis and purification of D(—)-2-(bromoacetyl)mandelic acid

Two methods were successfully employed for the synthesis of D(—)-2(bromoacetyl)mandelic acid. Method 1 [Methods 2.12.1 (a)], used for the synthesis of non-radiolabelled compound, involved the bromoacetylation of D(—)-mandelic acid by bromoacetylbromide in the presence of pyridine as a base catalyst (Figure 5.2). The reaction was monitored by TLC on silica gel plates, eluting with toluene/ethyl acetate/formic acid at 5:1:1 (v/v) and a typical synthesis is shown in Figure 5.3. The synthesis yielded two product compounds, D(—)-2-(bromoacetyl)mandelic acid which migrated with an R_f value of 0.43 (0.43, 0.43) and an unknown minor contaminant with an R_f value of 0.50 (0.50, 0.49). Both products were well separated from unreacted D(—)-mandelic acid [R_f = 0.14 (0.14, 0.13)] and pyridine which remained on the origin (R_f = 0).

When the solvent system was used for "flash" chromatography (Methods 2.12.2) it was again successful in separating the two products from unreacted D(—)-mandelic acid and pyridine, but it could not separate D(—)-2-(bromoacetyl)mandelic acid from the minor contaminant (Lane 4, Figure 5.3). Modifying the solvent system by using different ratios of toluene/ethyl acetate/formic acid resulted in loss of resolution. Formic acid was found to be essential as omission or an insufficient amount resulted in smearing, presumably because the carboxylate groups of the acids were not fully protonated. Furthermore, the amount of ethyl acetate could not be lowered significantly as an insufficient amount of ethyl acetate prevented the formic acid from being miscible in the solvent system. Therefore, several other TLC solvent systems were tested in an attempt to increase the separation between the two compounds. These included various combinations of chloroform/methanol, chloroform/methanol/formic acid, benzene or toluene/ethanol/formic acid and benzene or toluene/ethyl acetate/ethanol/formic acid, but none gave better separation than toluene/ethyl acetate/formic acid at 5:1:1 (v/v). All attempts at purifying D(—)-2-(bromoacetyl)mandelic acid to homogeneity using other techniques e.g. preparative TLC and crystallizing the compound from a number of single and double solvent systems also proved unsuccessful.

Method 2 [Methods 2.12.1 (b)], using bromo[1-^{14}C]acetic acid was developed for the synthesis of D(—)-2-(bromo[^{14}C]acetyl)mandelic acid as radiolabelled bromoacetylbromide was not commercially available. This involved a more complex two step reaction; in the first step, an anhydride was formed from the reaction of bromo[1-^{14}C]acetic acid with isobutylchloroformate in the presence of 4-methylmorpholine and then, in the second step, D(—)-mandelic acid was reacted with the anhydride to yield D(—)-2-(bromo[^{14}C]acetyl)mandelic acid, tertiary butanol and CO_2 as products (Figure 5.4). Although, in principle, the products should only have been those shown in Figure 5.4, again the same minor contaminant, as judged by its R_f value on TLC, appeared as with Method 1. After autoradiography of the TLC plate (Methods 2.2.8), both products were found to be radioactive although the number of counts incorporated into the contaminant was only about 9% of those incorporated into

D(—)-2-(bromo[^{14}C]acetyl)mandelic acid. However, despite the presence of the contaminant in the radioactive and non-radioactive preparations, preliminary inactivation experiments had shown that the product appeared suitable for further work.

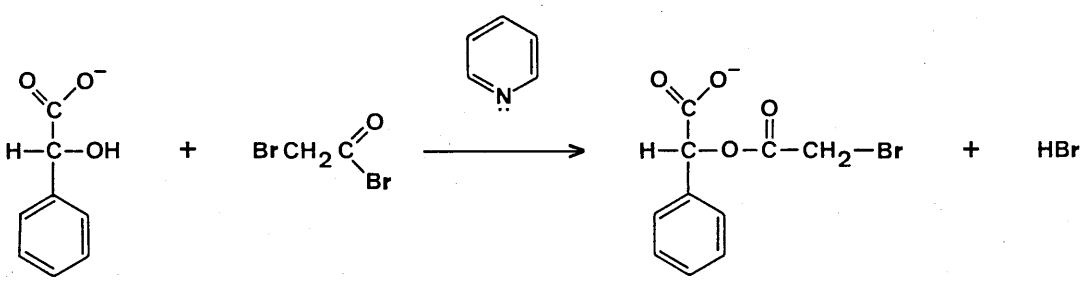


Figure 5.2 Synthesis of D(—)-2-(bromoacetyl)mandelic acid

Non-radiolabelled D(—)-2-(bromoacetyl)mandelic acid was synthesized from D(—)-mandelic acid and bromoacetylbromide in the presence of pyridine as a base catalyst as described in Methods 2.12.1 (a).

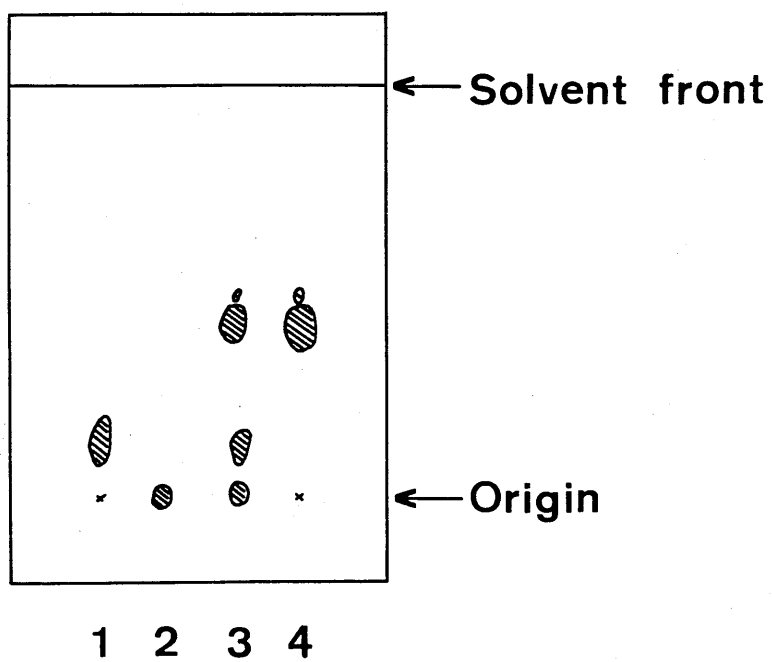


Figure 5.3 Synthesis and purification of D(—)-2-(bromoacetyl)mandelic acid (Method 1) monitored by TLC

The synthesis and purification of D(—)-2-(bromoacetyl)mandelic acid were monitored by TLC on silica gel plates, eluting with toluene/ethyl acetate/formic acid at 5:1:1 (v/v). The plates were then visualised under an ultra violet light source.

Lanes : 1, pure D(—)-mandelic acid; 2, pyridine; 3, crude product mixture; 4, purified D(—)-2-(bromoacetyl)mandelic acid and minor contaminant.

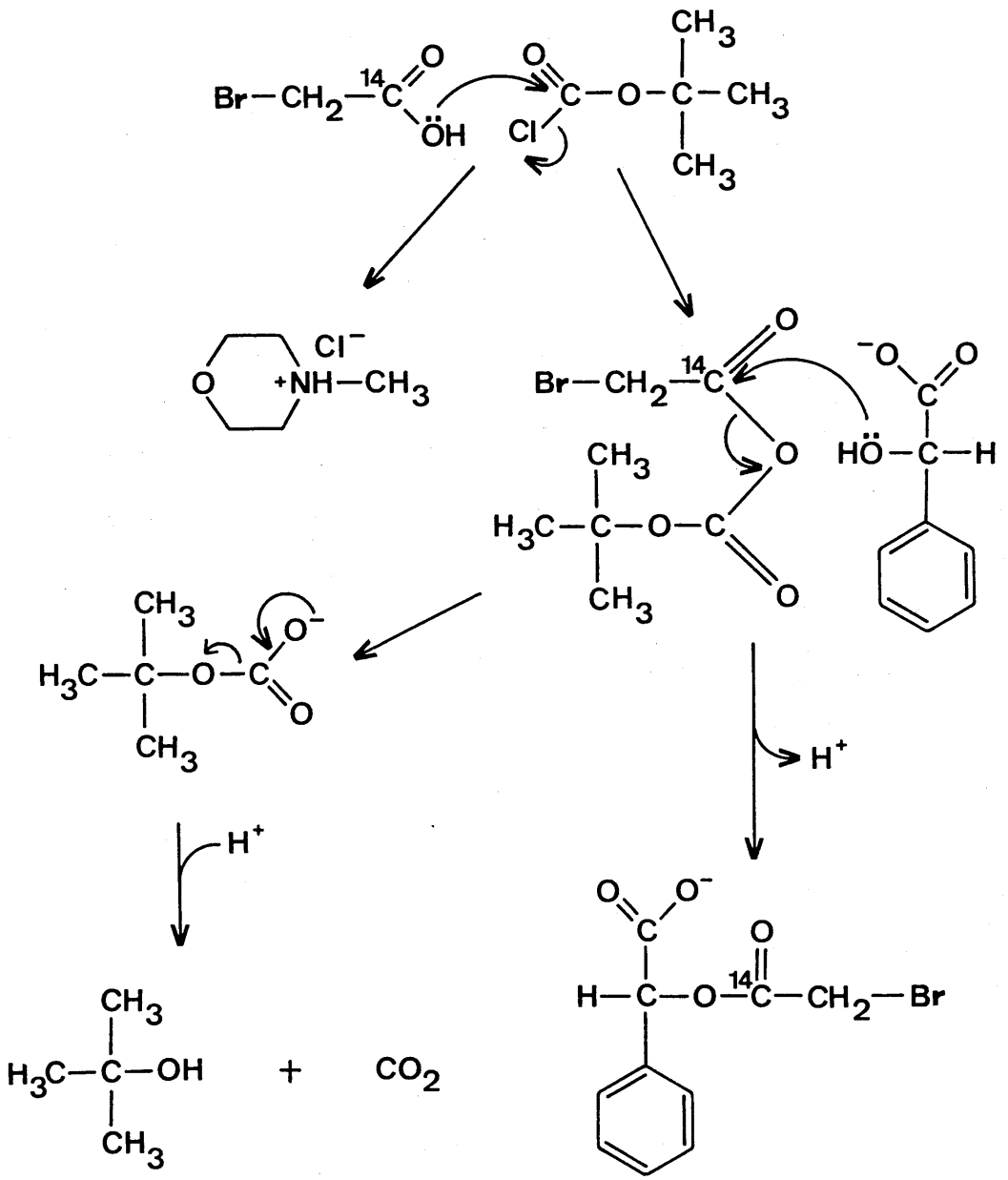


Figure 5.4 Synthesis of D(—)-2-(bromo[^{14}C]acetyl)mandelic acid

Radiolabelled D(—)-2-(bromoacetyl)mandelic acid was synthesized in a two step reaction which involved (1) formation of an anhydride from the reaction of bromo[1-^{14}C]acetic acid with isobutylchloroformate in the presence of 4-methylmorpholine, followed by (2) reaction of the anhydride with D(—)-mandelic acid to yield D(—)-2-(bromo[^{14}C]acetyl)mandelic acid, tertiary butanol and CO_2 as products, as described in Methods 2.12.1 (b).

5.3 Characterization of D(—)-2-(bromoacetyl)mandelic acid

The mass spectrum of D(—)-2-(bromoacetyl)mandelic acid confirmed the synthesis of the compound. The parent ions with mass to charge ratios of 272 and 274, corresponding to $C_{10}H_9O_4Br$ were detected (Peaks 1 & 2, Figure 5.5). Two peaks were detected as natural bromine exists as a diisotopic mix with ^{79}Br and ^{81}Br in an approximate 1:1 ratio, and therefore any molecular ion or fragment containing one bromine will give rise to a pair of peaks differing by 2 mass units (Williams & Fleming, 1973). Furthermore, high resolution analysis of the smaller parent ion gave a mass to charge ratio of 271.9684, exactly that required for $C_{10}H_9O_4Br$.

The ^1H-NMR spectrum (Figure 5.6) was also consistent with the structure of D(—)-2-(bromoacetyl)mandelic acid given in Figure 5.1. The resonances, relative to chloroform at 7.25 ppm, were at 3.96 ppm (2 H, singlet; methylene protons), 6.0 ppm (1 H, singlet; HC-proton), 7.39 - 7.52 ppm (5 H, multiplet; aromatic protons) and 12.01 ppm [1 H, singlet; consistent with an acidic proton resonating at very low field strength (Abraham & Loftus, 1978)]. There was also a resonance at 3.85 ppm (singlet) which presumably represented a signal from the contaminant, and a resonance at 1.97 ppm (singlet), consistent with the signal from acetonitrile (Aldrich, 1983), indicating that residual solvent from the purification had remained even after extensive lyophilization. Confirmation that the resonance at 3.96 ppm were those of the methylene protons was obtained after analysis of the spectrum in the presence of benzene (25% v/v). As the methylene group forms part of the bromoacetyl side chain which is bonded to a chiral carbon, the two methylene protons experience different magnetic fields and are therefore diasterically not equivalent. As a consequence, they resonate at slightly different frequencies, although in the presence of deuterochloroform alone this difference is insufficient to be detected as two separate signals. However, in the presence of benzene, which can increase the diasteriotopic effect by slowing down the rotation of the methylene carbon, the methylene proton resonances were detected as discrete signals at 3.563 ppm and 3.559 ppm relative to benzene at 7.15 ppm as is shown in Figure 5.6, inset. It was also apparent that the methylene protons resonated at higher frequency in the presence of benzene, although solvents containing aromatic groups such as benzene and pyridine may cause large changes in the observed chemical shifts of the solute when compared with the spectra run in non-aromatic solvents (Derome, 1987).

The elemental composition of D(—)-2-(bromoacetyl)mandelic acid was also consistent with the analytical calculation for $C_{10}H_9O_4Br$:

Element	Expected (%)	Found (%)
Carbon	44.1	42.6 (42.6, 42.6)
Hydrogen	3.3	2.7 (2.6, 2.8)
Bromine	29.0	30.4 (single expt.)
Oxygen	23.5	not determined

The CD spectrum of D(—)-2-(bromoacetyl)mandelic acid was analysed to determine the stereochemistry of the compound relative to that of D(—)- and L(+)-mandelic acid (Figure 5.7). D(—)-Mandelic acid gave a negative cotton effect, resulting in a maximum at 223 nm whereas L(+)-mandelate gave the positive mirror image. D(—)-2-(Bromoacetyl)mandelic acid also gave a negative cotton effect with an identical maximum to D(—)-mandelic acid but at approximately 220 nm, and therefore it had the same stereochemistry as D(—)-mandelic acid, the precursor used for its synthesis.

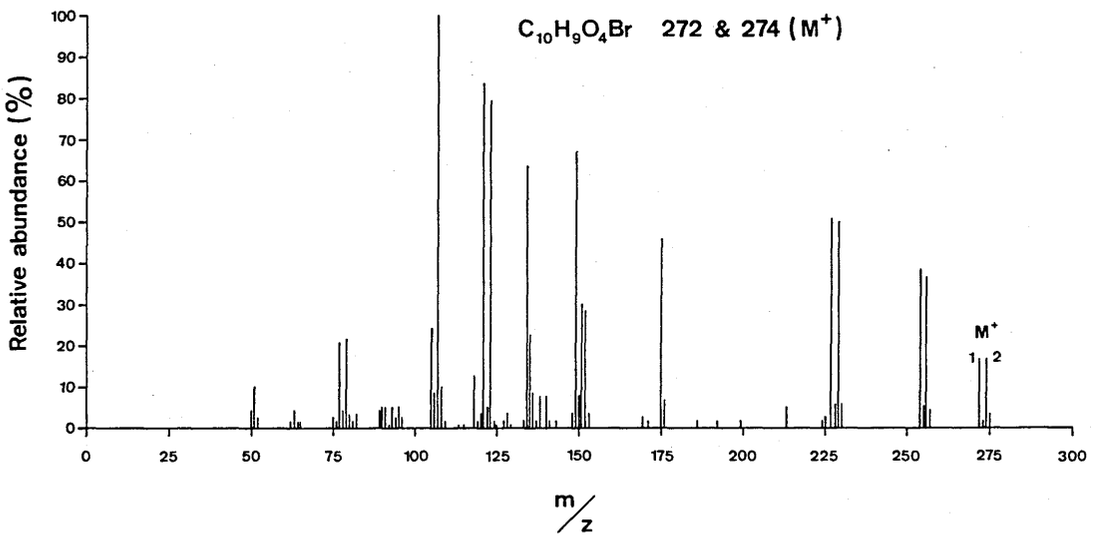


Figure 5.5 Mass spectrum of D(—)-2-(bromoacetyl)mandelic acid

The mass spectrum of D(—)-2-(bromoacetyl)mandelic acid was obtained using electron impact mass spectrometry with a Kratos MS-30 mass spectrometer by Dr T. Bugg and colleagues, Department of Organic Chemistry, University of Cambridge.

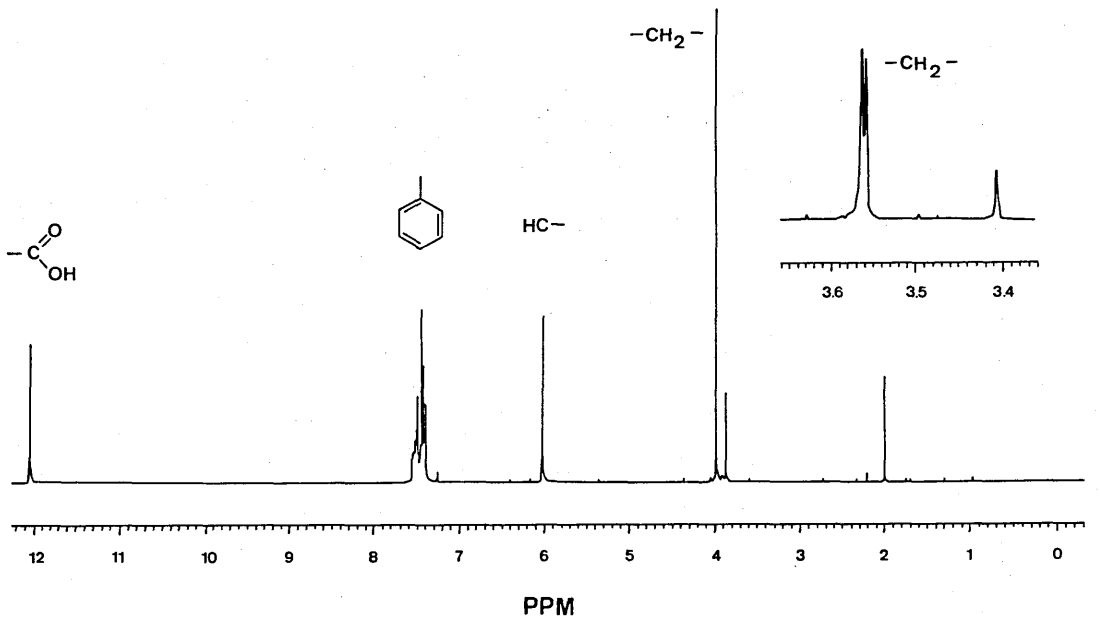


Figure 5.6 ^{1}H-NMR spectrum of D(—)-2-(bromoacetyl)mandelic acid

The ^{1}H-NMR spectrum of D(—)-2-(bromoacetyl)mandelic acid was obtained using a Bruker model WP 220 SY (200 MHz) NMR spectrometer, by Mr J. Gall, Department of Chemistry, University of Glasgow. The compound was dissolved in deuterochloroform and the resonances referenced to chloroform at 7.25 ppm (for the main spectrum) and benzene [25% (v/v) in deuterochloroform] at 7.15 ppm for the inset spectrum.

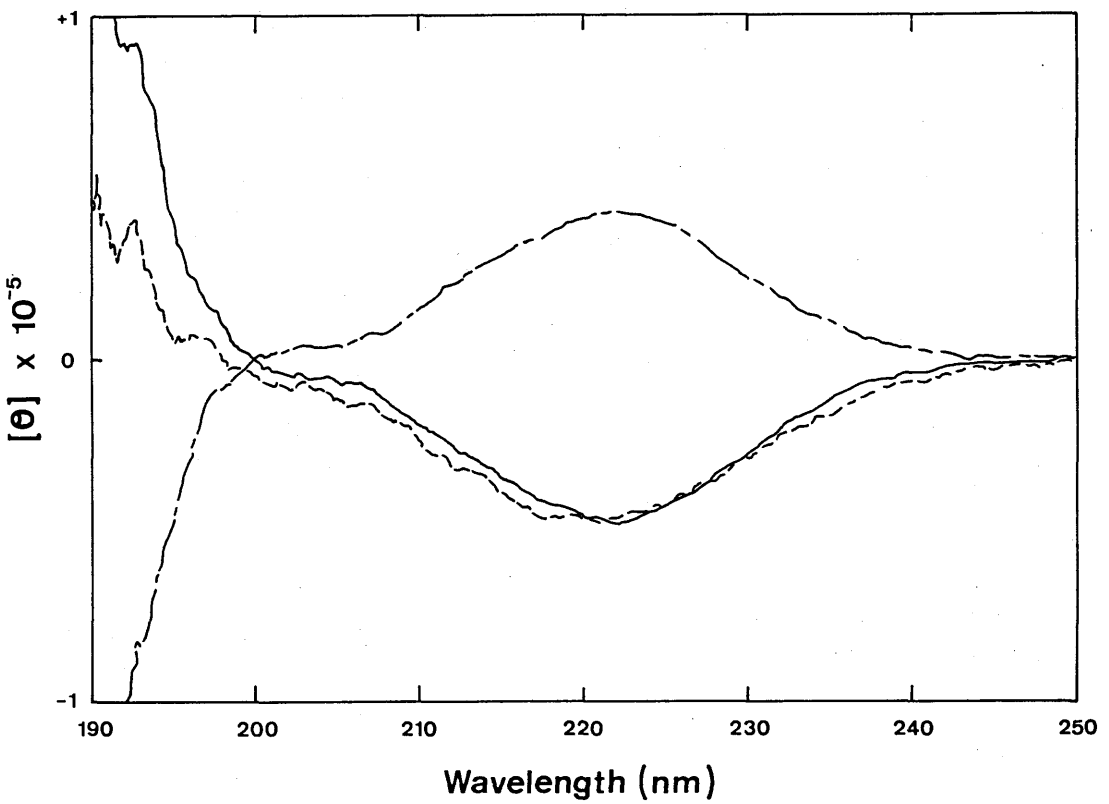


Figure 5.7 CD spectrum of D(—)-2-(bromoacetyl)mandelic acid, D(—)-mandelic acid and L(+)-mandelic acid

Circular dichroism was carried out by Ms S. Kelly using a JASCO J-600 Spectropolarimeter at the Scottish CD Facility, School of Biological Sciences, University of Stirling. D(—)-2-(Bromoacetyl)mandelic acid (0.2 mg ml^{-1}, 0.74 mM) and D(—)- and L(+)-mandelic acids (0.2 mg ml^{-1}, 1.3 mM) dissolved in acetonitrile (HPLC grade), were scanned from 190-250 nm in a 0.02 cm pathlength cuvette. D(—)-mandelic acid (———), L(+)-mandelic acid (—— — ——) and D(—)-2-(bromoacetyl)mandelic acid (— — —).

5.4 Kinetics of inactivation of D(—)-mandelate dehydrogenase by D(—)-2-(bromoacetyl)mandelate

In preliminary experiments, D(—)-mandelate dehydrogenase was found to be inactivated by D(—)-2-(bromoacetyl)mandelate, and incubation of the enzyme with 8 mM-D(—)-2-(bromoacetyl)mandelate typically resulted in >95% inactivation after 2 h at 27°C. When the enzyme was incubated under the same conditions in the absence of D(—)-2-(bromoacetyl)mandelate but in the presence of 0.95 M-acetonitrile (the concentration in the inactivation mix), the enzyme remained fully active indicating that the solvent alone did not inhibit enzymic activity. Moreover, when enzyme, inactivated with D(—)-2-(bromoacetyl)mandelate, was dialysed extensively against 50 mM-Tris/HCl buffer (pH 8.0), there was no restoration of enzymic activity indicating that modification of the enzyme was probably covalent.

The inactivation of D(—)-mandelate dehydrogenase with D(—)-2-(bromoacetyl)mandelate in the absence and presence of D(—)-mandelate, followed pseudo-first-order kinetics. The half-times of inactivation were measured for 5 different concentrations of inhibitor, and the curves obtained are shown in Figure 5.8. A plot of half-time of inactivation ($t_{1/2}$) against the reciprocal D(—)-2-(bromoacetyl)mandelate concentration according to the equation (Meloche, 1967):

$$t_{1/2} = 1/([\text{D(—)-2-(bromoacetyl)mandelate}]\, t_{min}\, K_i) + t_{min}$$

gave two straight lines with positive ordinate intercepts, indicating that the inactivation of D(—)-mandelate dehydrogenase obeyed rate saturation kinetics (Figure 5.9). The inhibitor therefore forms a dissociable complex with the enzyme prior to inactivation, since the secondary plot in the absence of D(—)-mandelate has an intercept giving a finite inactivation half-time (t_{min}) of 22.7 min (21.1, 24.3), and the inhibitor concentration giving the half-maximal rate of inactivation (K_i) was 2.4 mM (2.4, 2.3) (See Figure 5.9).

The affinity of D(—)-2-(bromoacetyl)mandelate for the substrate binding site/active site was demonstrated by the increase in the slope of the secondary plot when the enzyme was inactivated in the presence of D(—)-mandelate. This resulted in a half-maximal rate of inactivation (K_i) of 4.8 mM i.e. the apparent affinity for the inhibitor decreased in the presence of competing substrate (Kitz & Wilson, 1962). The ordinate intercept increased slightly (See Figure 5.9), resulting in a finite inactivation half-time (t_{min}) of 29.1 min, although in other experiments the ordinate intercepts of secondary plots in the absence and presence of D(—)-mandelate remained unchanged.

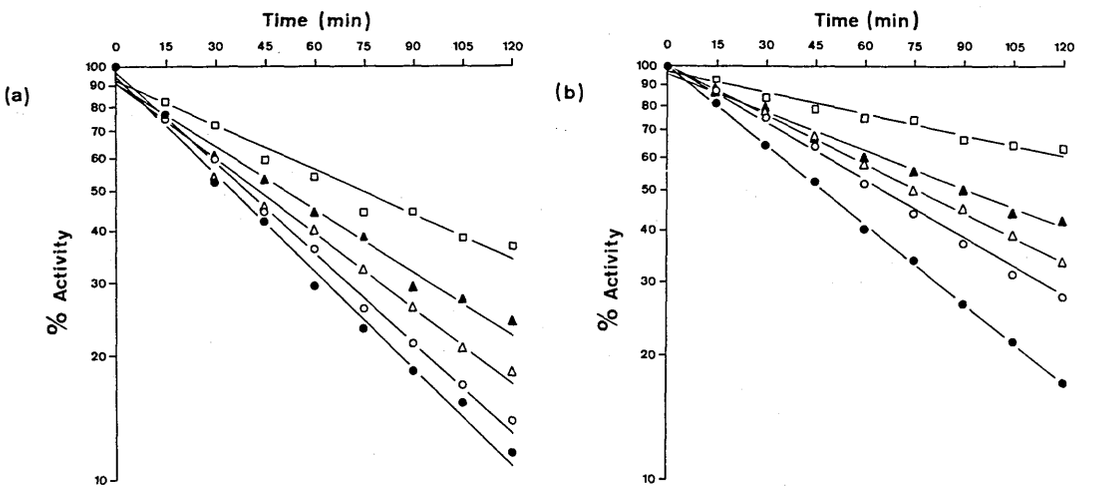


Figure 5.8 Inactivation of D(—)-mandelate dehydrogenase by various concentrations of D(—)-2-(bromoacetyl)mandelate in the absence and presence of D(—)-mandelate

(a) Inactivation of D(—)-mandelate dehydrogenase in the absence of D(—)-mandelate. The inactivation mix (100 μl) contained D(—)-mandelate dehydrogenase (19 μg, 5 μM), D(—)-2-(bromoacetyl)mandelate (1-4 mM) and acetonitrile (0.95 M). Immediately after addition of the label, the inactivation mix was vortexed and a sample (10 μl) removed and diluted into 990 μl of 50 mM-Tris/HCl (pH 8.0). Samples (10-40 μl) were then assayed for residual phenylglyoxylate reductase activity as in Methods 2.4.2 (c). At 15 min intervals throughout the 2 h time course (27°C), samples (10 μl) were removed, diluted and assayed for residual enzymic activity. The activity of the sample taken immediately after addition of the label was taken as the 100% value. Concentrations of D(—)-2-(bromoacetyl)mandelate : 4 mM (●), 3 mM (○), 2 mM (△), 1.5 mM (▲) and 1 mM (□).

(b) Inactivation of D(—)-mandelate dehydrogenase in the presence of D(—)-mandelate. The inactivation was carried out exactly as described above except that the inactivation mix also contained 5 mM-D(—)-mandelate.

Concentrations of D(—)-2-(bromoacetyl)mandelate : 8 mM (●), 4 mM (○), 3 mM (△), 2 mM (▲) and 1 mM (□).

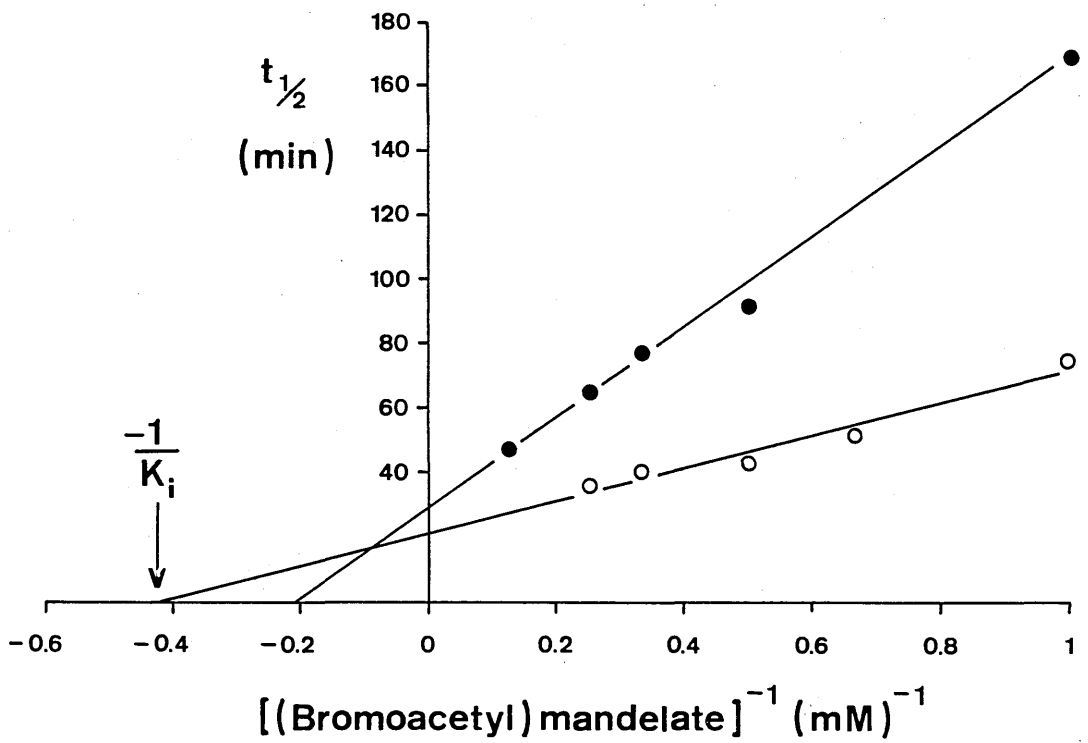


Figure 5.9 Secondary plots of half-time of inactivation ($t_{1/2}$) as a function of the reciprocal D(—)-2-(bromoacetyl)mandelate concentration

The half-times of inactivation of D(—)-mandelate dehydrogenase at several concentrations of D(—)-2-(bromoacetyl)mandelate were determined from the primary plots shown in Figure 5.8. The enzyme was inactivated in the presence of inhibitor in the absence (○) and presence (●) of 5mM-D(—)-mandelate.

5.5 Stoicheiometry of Inactivation

When D(—)-mandelate dehydrogenase was incubated with D(—)-2-(bromo[^{14}C]acetyl)mandelate, radioactivity was incorporated into the protein. The incorporation of radioactivity into the enzyme followed a linear relationship with the concomitant loss of enzymic activity (Figure 5.10). The enzyme was inactivated to 12.5% remaining activity and extrapolation of the line of best fit to total inactivation gave a stoicheiometry of 1.27 moles of inhibitor bound per mole of enzyme i.e. a value approximating to a stoicheiometry of 1:1, indicating that the inhibitor reacts at a single site on the enzyme although some secondary sites are also reacting before all of the active sites are modified.

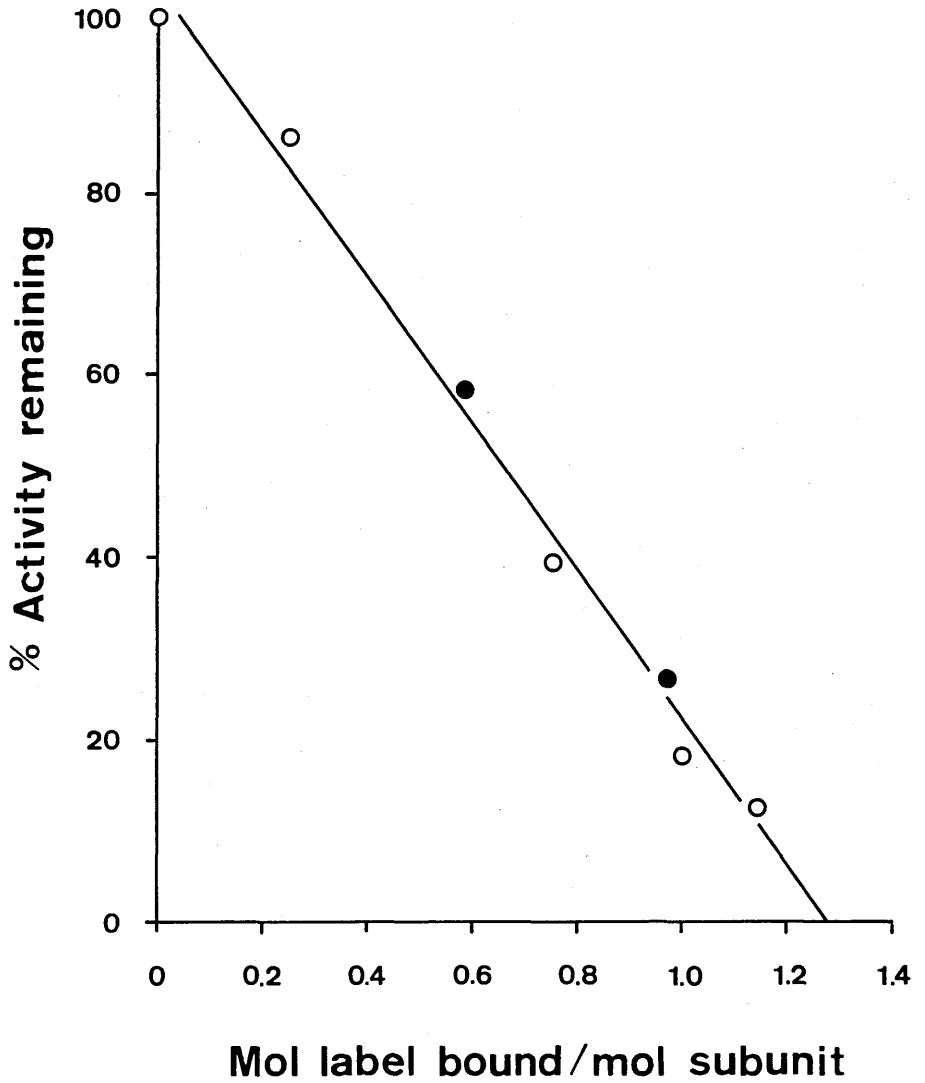


Figure 5.10 Incorporation of D(—)-2-(bromo[^{14}C]acetyl)mandelate into D(—)-mandelate dehydrogenase as a function of the residual enzymic activity

The inactivation mix (1125 μl) contained D(—)-mandelate dehydrogenase (1.3 mg, 30.4 μM), D(—)-2-(bromo[^{14}C]acetyl)mandelate (1.4 mM) and acetonitrile (420 mM). Immediately after addition of the label, the inactivation mix was vortexed and a sample (5 μl) removed and diluted into 995 μl of 50 mM-Tris/HCl (pH 8.0). This was further diluted (250 μl into 750 μl of the same buffer) before samples (10 μl) were assayed for phenylglyoxylate reductase activity as in Methods 2.4.2 (c). At intervals throughout the 3 h inactivation, 5 μl were removed, diluted and appropriate samples (10-100 μl) assayed for residual activity. The activity of the sample taken immediately after addition of the label was taken as the 100% value. Covalent incorporation of label was monitored by a filter disc method (○) and gel filtration (●) as described in Methods 2.13.2.

5.6 Identification and purification of active site labelled peptides

Before D(—)-mandelate dehydrogenase was inactivated for peptide purification, preliminary experiments were carried out to optimise peptide separation on reverse-phase HPLC. D(—)-Mandelate dehydrogenase peptides were separated initially with a gradient system of 100% A (0.1% TFA in water) to 100% B (0.1% TFA in acetonitrile) in 60 min, but in subsequent experiments this was modified to shallower gradients of 100% A to 70% B and later to 90% A to 50% B over the same time scale (Methods 2.15.2), which resulted in better separation, especially of the early eluting peptides, without significant loss of resolution. Recovery of radiolabel from the column was generally 70%-90% of the total counts loaded.

The location of the active site labelled peptides was determined by substrate protection experiments, where the peptide profiles and distribution of radiolabel from enzyme inactivated in the absence of D(—)-mandelate were compared with those from enzyme inactivated in the presence of D(—)-mandelate. As D(—)-mandelate protects the enzyme from inactivation by D(—)-2-(bromoacetyl)mandelate (Section 5.4), incorporation of radiolabel into the active site peptides should be reduced in the presence of the substrate. Two samples of D(—)-mandelate dehydrogenase (500 μg, 13.2 nmol) were inactivated with D(—)-2-(bromo[^{14}C]acetyl)mandelate, one in the absence of substrate and the other in the presence of substrate [Methods 2.13.3 (a,b)]. After incubation for 4 h at 27°C the unprotected sample had lost 76% of its original activity, whilst the protected sample had lost only 27%. After gel filtration, NaOH treatment and trypsin digestion [Methods 2.13.3 (d) & 2.15.1] the peptides were separated by reverse-phase HPLC (system I, Methods 2.15.2) and the location of radiolabel determined by scintillation counting (Methods 2.2.8). Comparison of the HPLC chromatographs (Figure 5.11) shows that for an equal loading of protein, incorporation of radiolabel into tryptic peptides 1 (TP 1) and 2 (TP 2) was clearly reduced in the presence of the substrate. Once the location of the labelled active site peptides had been determined, the enzyme was inactivated on a larger scale for peptide purification and sequencing. Initially, 2.8 mg (73.7 nmol) of D(—)-mandelate dehydrogenase was inactivated, but there was only sufficient material to purify enough TP 1 for a single sequencing run. TP 2 could not be purified, not only because of lack of material but also because the resolution of the column (column 1) had deteriorated after extensive use. Therefore, a new column (column 2) was used and a fresh batch of enzyme purified for inactivation.

D(—)-Mandelate dehydrogenase (5 mg, 132 nmol) was inactivated to 15% remaining activity with D(—)-2-(bromo[^{14}C]acetyl)mandelate [Methods 2.13.3 (c)] and, after gel filtration, NaOH treatment and trypsin digestion [Methods 2.13.3 (d) & 2.15.1], the peptides were separated on reverse-phase HPLC (System I, Methods 2.15.2). Prior to large scale injection, a small sample was run on column 2 to check that the radiolabelled peptides eluted at the same relative positions in the profile as they did from column 1, which had been used to identify their location. Figure 5.12 shows the HPLC chromatograph of this sample, and comparison with the elution profiles given in

Figure 5.11 (column 1) clearly shows that the resolution of the new column was better, and although the absolute positions at which TP 1 and TP 2 eluted were slightly different from that of the first column, their relative positions in the profile remained essentially unchanged. After preparative injection (13.2 nmol) of D(—)-mandelate dehydrogenase peptides, the peaks corresponding to TP 1 and TP 2 were collected manually and, in addition, a third peptide (TP 3) was also collected for sequencing (Figure 5.12). TP 3 was the last to elute from the column, suggesting that it was hydrophobic in nature, and was sufficiently separated from neighbouring peptides that further purification was unnecessary. The pooled fractions from system I containing TP 1 were lyophilized, resuspended in water and the peptides separated using solvent system II (Methods 2.15.2, Figure 5.13). The peak corresponding to radiolabel was collected manually and, after multiple injections, the fractions were pooled and lyophilized. Pure TP 1 was resuspended in water and desalted using solvent system I modified from 60 min to 25 min (Methods 2.15.2, Figure 5.14), and again, after multiple injections, the pooled fractions were finally lyophilized for sequencing.

When attempts were made to purify TP 2 further using system II, it co-eluted with a number of other peptides and even modified gradients failed to separate it from the contaminants. Better separation was achieved when TP 2 was eluted using system III (Methods 2.15.2). However, unlike TP 1 it could not be purified to homogeneity even though a number of modified gradients were again tested. The best separation achieved is shown in Figure 5.15 and although the counts were spread, making it impossible to assign the radiolabel to any one peptide, fractions 8 and 9 were pooled and lyophilized. The pooled fractions from system III containing TP 2 were then resuspended in water and desalted with a shallow system I gradient (70% A to 60% B in 30 min, Methods 2.15.2), which resulted in the co-elution of two peptides as indicated by a distinctive shoulder to the main peak (Figure 5.16).

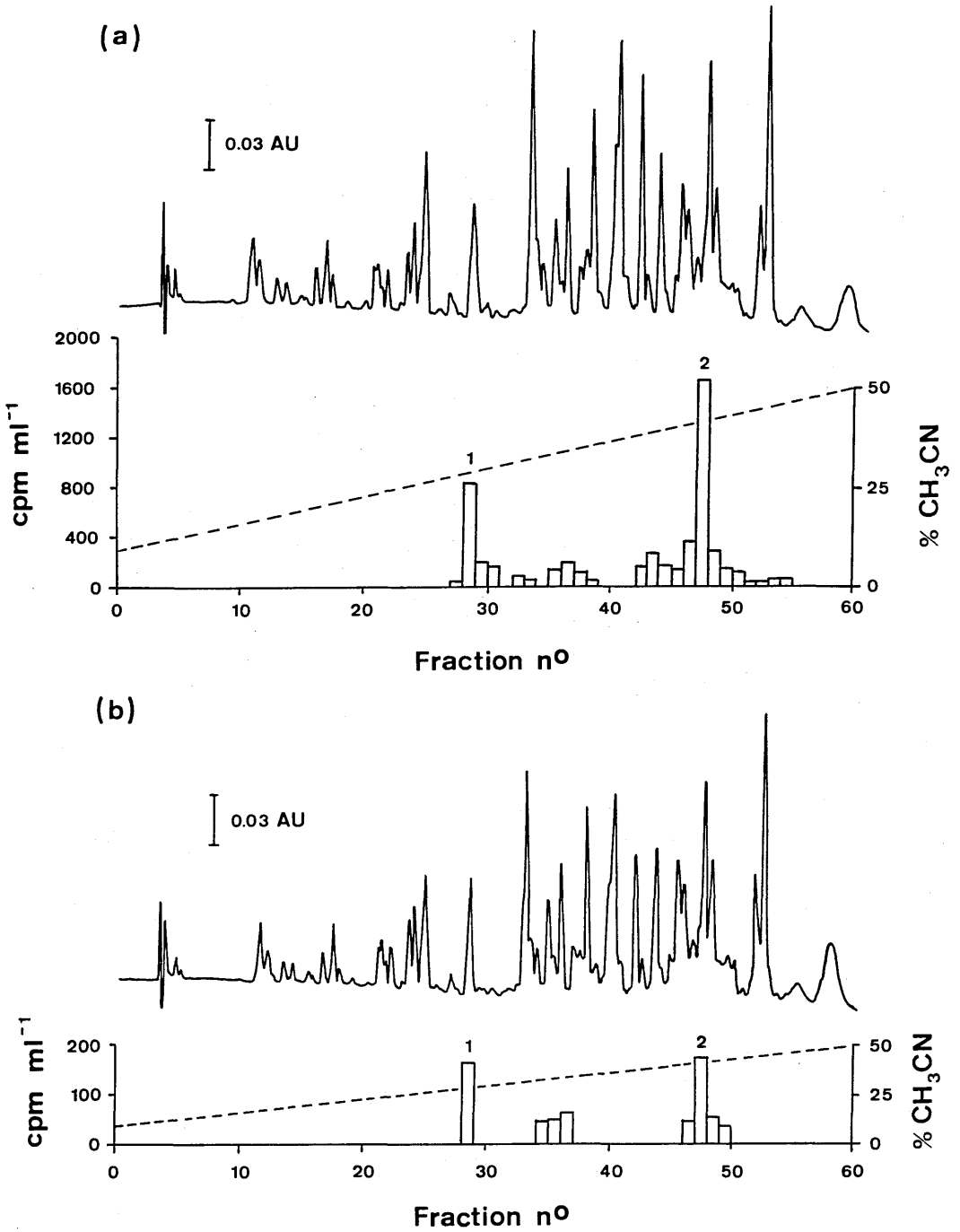


Figure 5.11 Reverse-phase HPLC chromatographs of tryptic digests of D(—)-mandelate dehydrogenase inactivated in the absence and presence of D(—)-mandelate (column 1)

Tryptic peptides (5.2 nmol) were prepared as in Methods 2.13.3 (d) and 2.15.1 from enzyme inactivated with D(—)-2-(bromo[^{14}C]acetyl)mandelate in (a) the absence and (b) the presence of D(—)-mandelate [Methods 2.13.3 (a) & (b) respectively]. The peptides were then separated by reverse-phase HPLC with an acetonitrile gradient of 90% A (0.1% TFA in water) to 50% B (0.1% TFA in acetonitrile) in 60 min. Fractions (1 ml) were collected and the location of radiolabel was determined by scintillation counting as described in Methods 2.2.8.

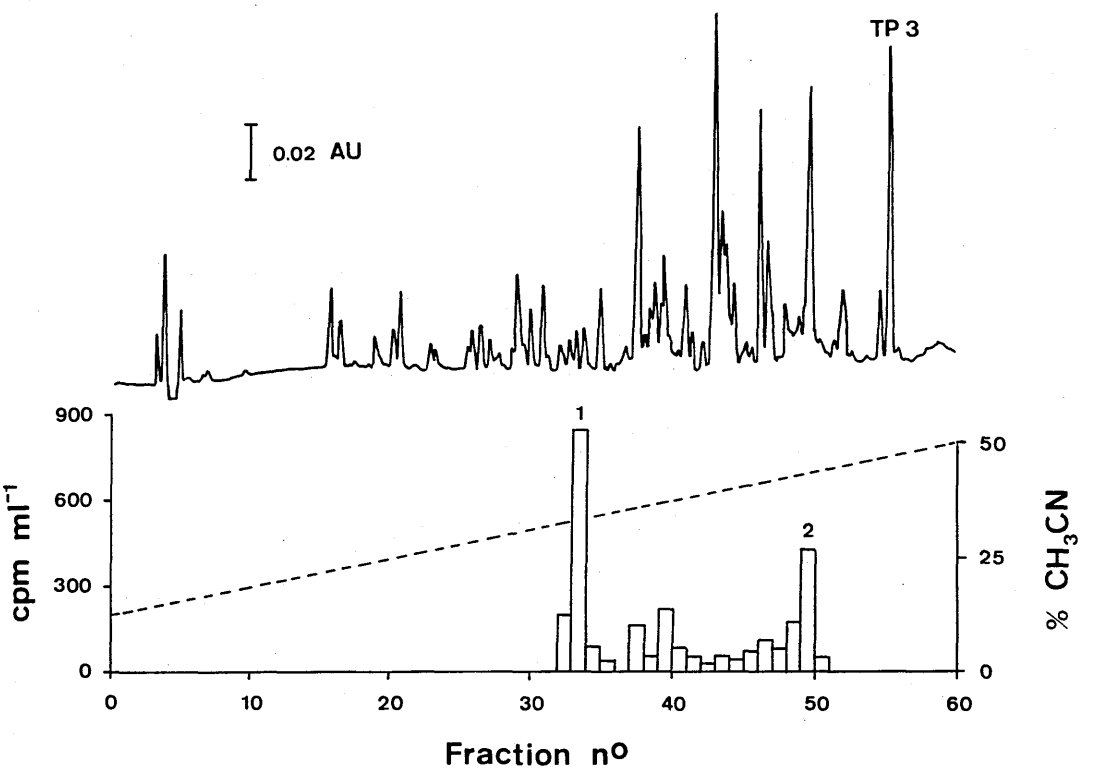


Figure 5.12 Reverse-phase HPLC chromatograph of a tryptic digest of inactivated D(—)-mandelate dehydrogenase (column 2)

Tryptic peptides (2.5 nmol) prepared as in Methods 2.13.3 (d) and 2.15.1 from enzyme inactivated with D(—)-2-(bromo[^{14}C]acetyl)mandelate [Methods 2.13.3 (c)], were separated by reverse-phase HPLC with an acetonitrile gradient of 90% A (0.1% TFA in water) to 50% B (0.1% TFA in acetonitrile) in 60 min. The location of radiolabel was determined by scintillation counting (Methods 2.2.8). During large scale injection (13.2 nmol), the peaks corresponding to TP 1, TP 2 and TP 3 were collected manually.

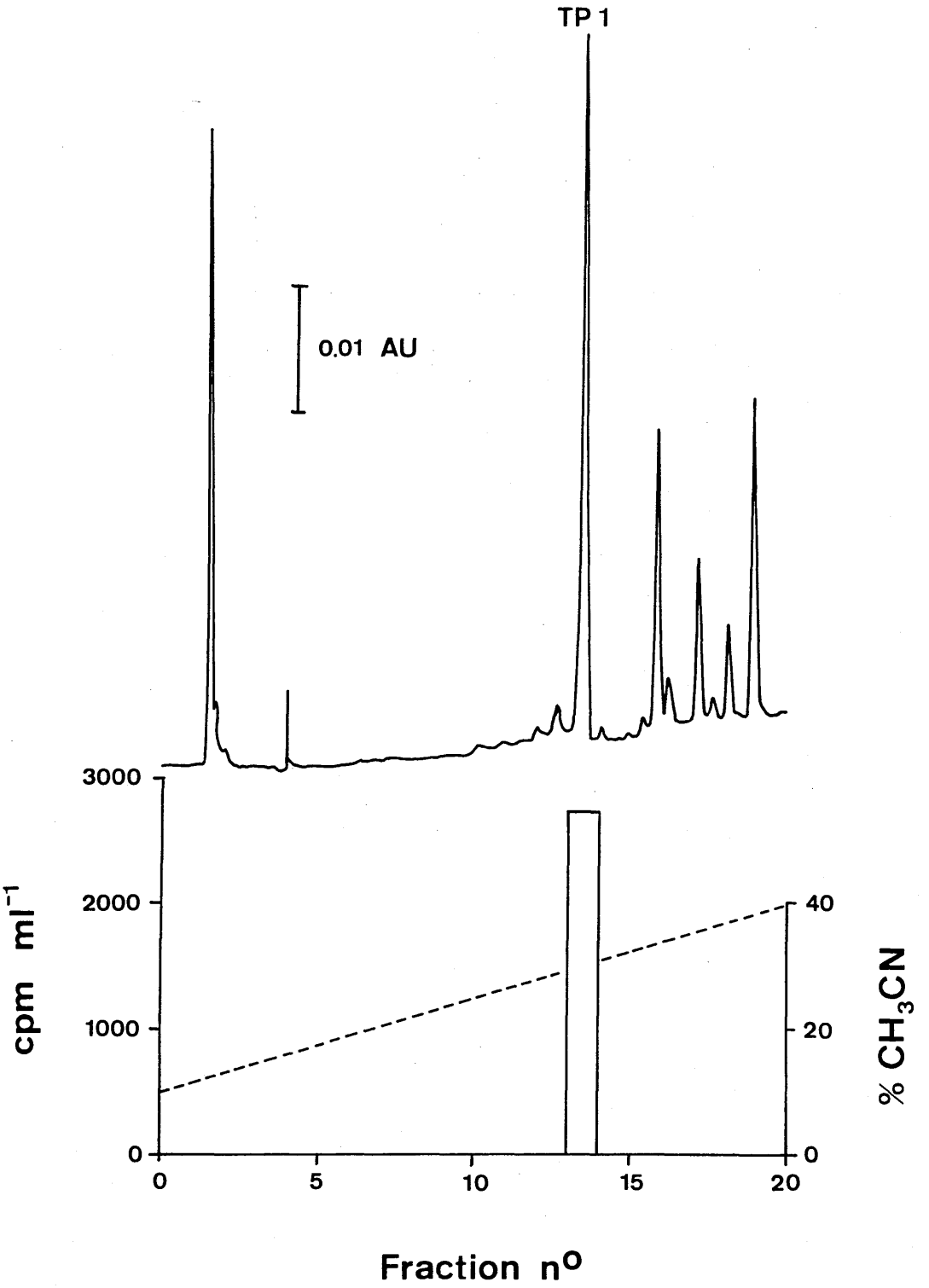


Figure 5.13 Purification of TP 1 by reverse-phase HPLC (column 2)

The pooled fractions from system I containing TP 1 were lyophilized, resuspended in water and the peptides separated with an acetonitrile gradient of 90% A [1 mM-sodium phosphate (pH 7.4)] to 40% B (acetonitrile) in 20 min.

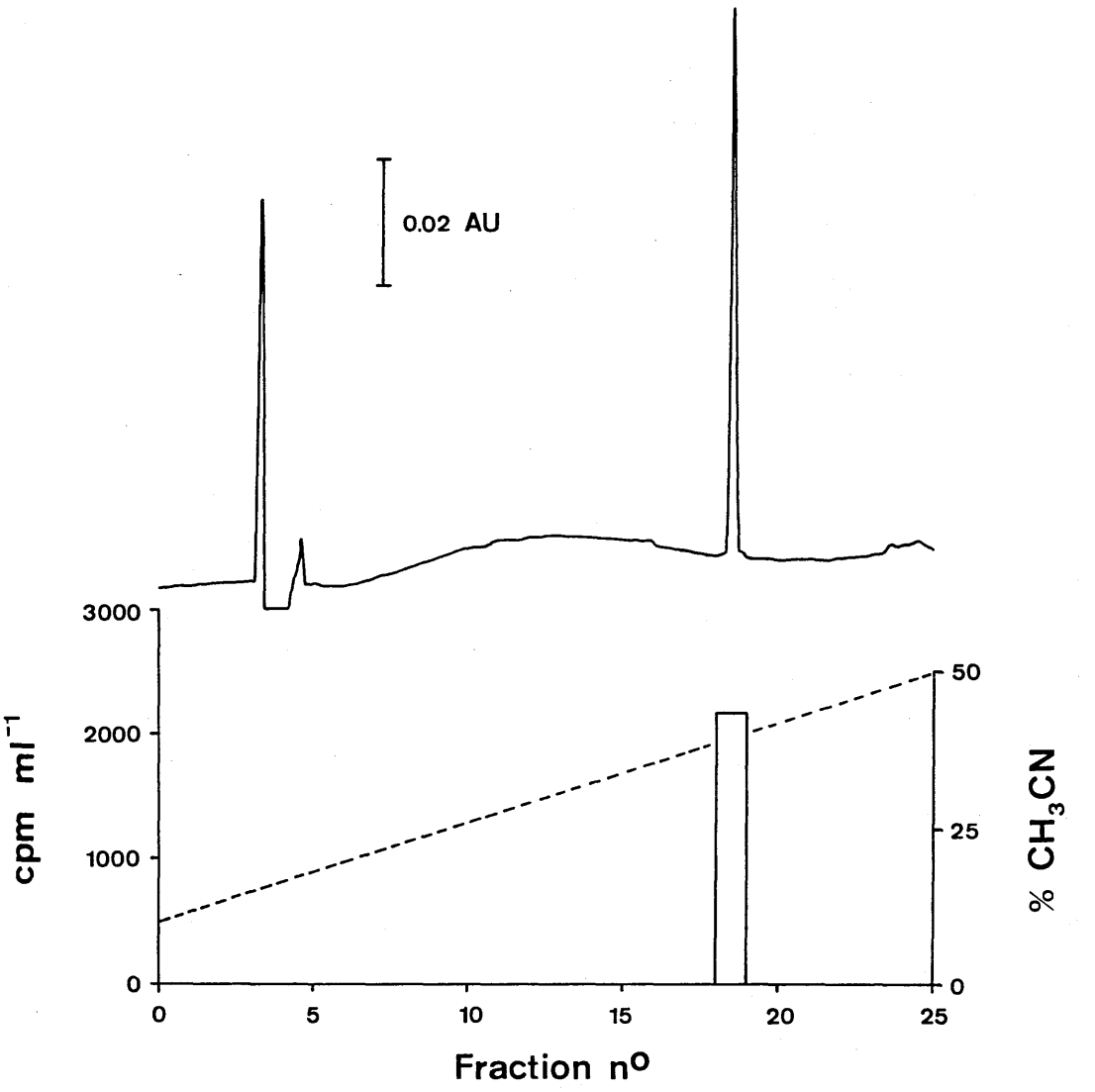


Figure 5.14 Desalting TP 1 by reverse-phase HPLC (column 2)

Fractions from system II containing pure TP 1 were lyophilized, resuspended in water and desalted with an acetonitrile gradient of 90% A (0.1% TFA in water) to 50% B (0.1% TFA in acetonitrile) in 25 min.

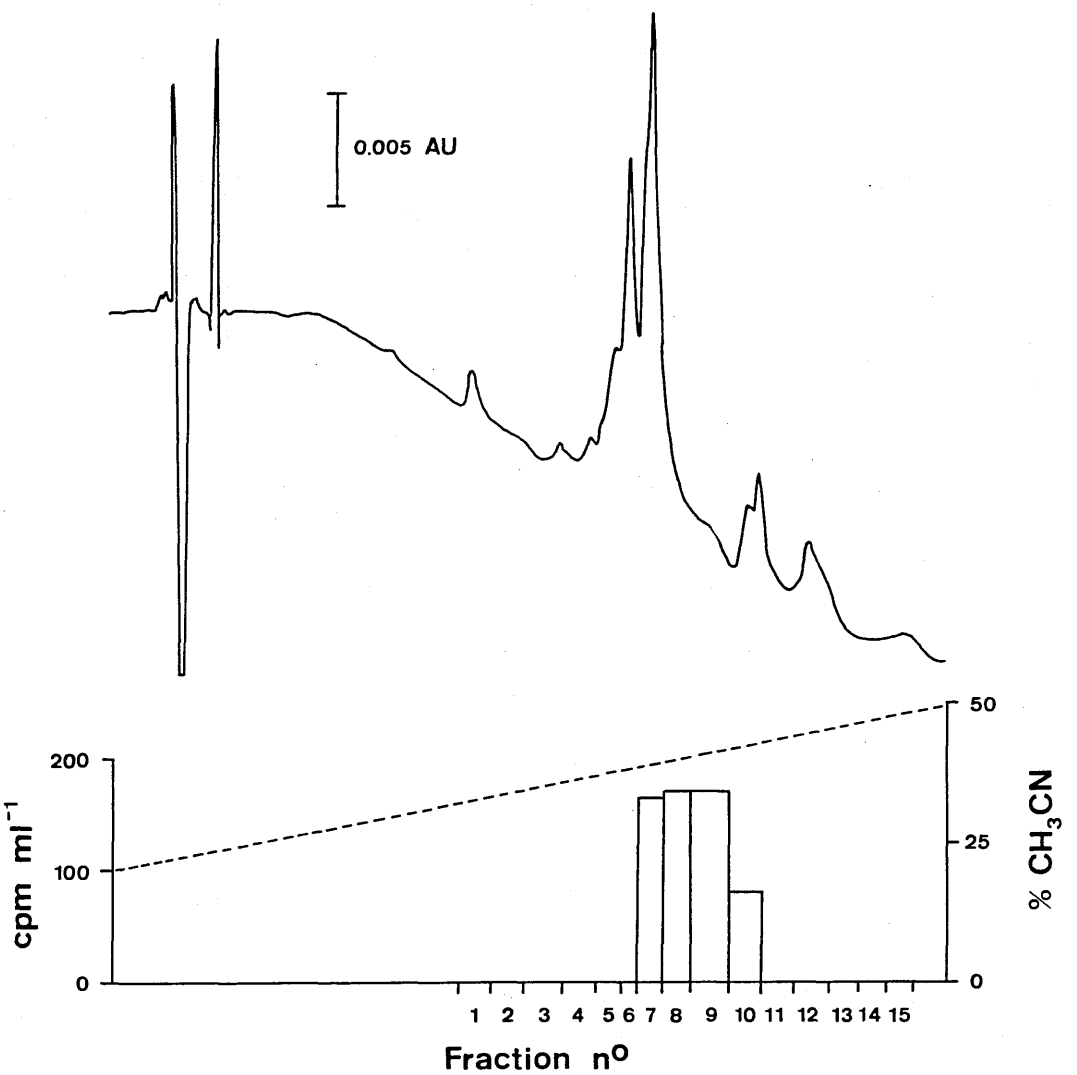


Figure 5.15 Partial purification of TP 2 by reverse-phase HPLC (column 2)

The pooled fractions from system I containing TP 2 were lyophilized, resuspended in water and the peptides separated with an acetonitrile gradient of 80% A [0.05% ammonium acetate (pH 6.0)] to 50% B (acetonitrile) in 30 min.

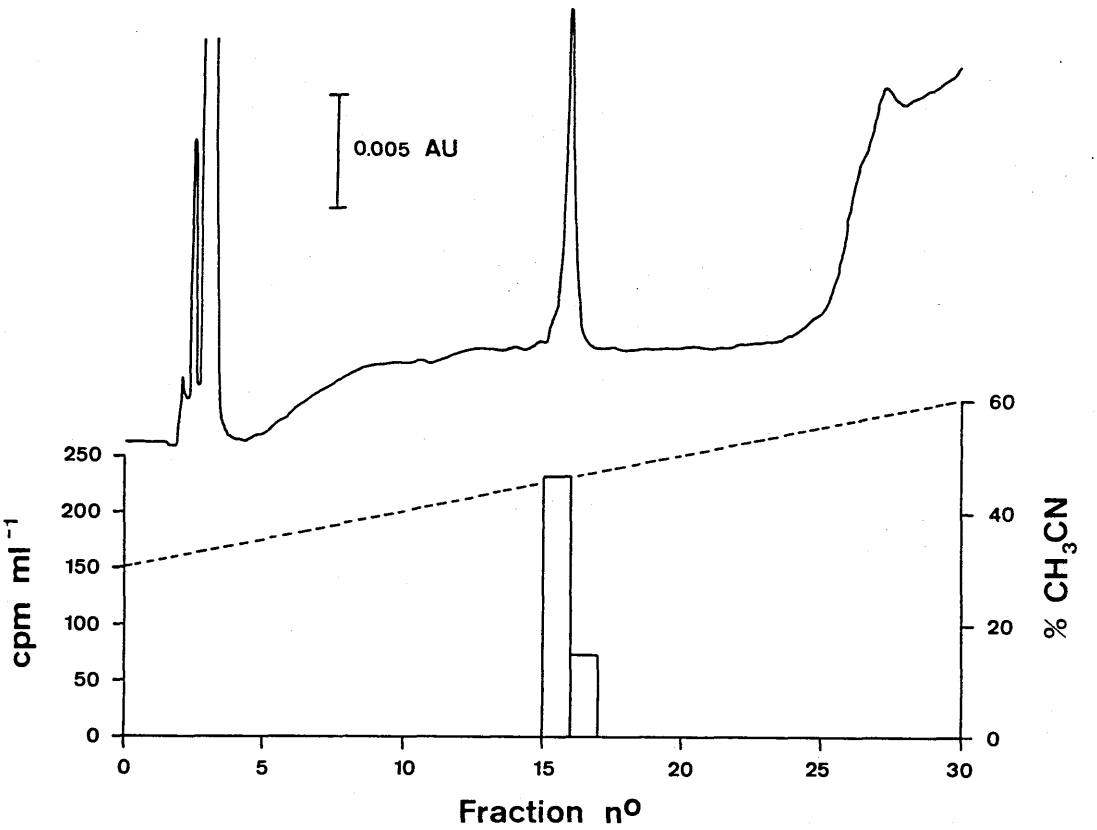


Figure 5.16 Desalting TP 2 by reverse-phase HPLC (column 2)

Fractions from system III containing partially pure TP 2 were lyophilized, resuspended in water and desalted with an acetonitrile gradient of 70% A (0.1% TFA in water) to 60% B (0.1% TFA in acetonitrile) in 30 min.

5.7 Peptide sequencing

The amino acid composition and sequence of TP 1 were first determined at the Department of Biochemistry, University of Dundee. The amino acid composition (Methods 2.15.4) indicated that TP 1 was composed of 7 different types of amino acid : Glx, Gly, Val, Ile, Leu, Lys and an unidentified acidic residue. The sequence of TP 1 (Table 5.1) confirmed the amino acid composition and, moreover, showed that the Glx identified by amino acid analysis was in fact a glutamate. The peptide was found to be 7 residues in length although the second residue, presumably the modified amino acid, could not be identified. Although sufficient peptide was supplied for sequencing and detection of radiolabel (2 nmol, 4000 cpm) no counts above the background level were detected in any of the fractions eluting from the sequencer.

A larger sample of TP 1 (4 nmol, 8000 cpm), along with TP 2 (approx. 1 nmol, 1250 cpm) and TP 3 (approx. 100 nmol) were then sequenced at the SERC Sequencing Unit, Department of Biochemistry, University of Leeds. In an attempt to identify the position of the modified/radiolabelled residue in TP 1, the peptide was sequenced using solid-phase and liquid-pulse sequencing (Methods 2.15.6 and 2.15.7). The sequences and recovery of radiolabel are given in Table 5.2. The unidentified residue at position 2 eluted from the column between the standards aspartic acid and *S*-carboxymethylcysteine, confirming its acidic nature, but also indicating that the single cysteine residue identified by amino acid analysis (Section 4.2.2) is not modified by D(—)-2-(bromoacetyl)mandelate. In addition to the unknown residue at position 2, solid-phase sequencing could neither identify the amino terminal residue nor the lysine at position 7 as they remained bound to the support matrix after the degradation cycle, via their α-amino and ε-amino groups respectively. The radiolabel eluted with the unidentified residue at position 2 although the counts were too low to be more than indicative of this residue being labelled. However, more significant counts were detected using the liquid-pulse sequencer, and although there was a spread of radiolabel in several fractions, it did not begin to elute until the second residue had been cleaved. In the light of both these results it seems likely that the radiolabel has been incorporated into the residue at position 2 although its identity remains unknown. The sequence of TP 1 purified with column 1 was also determined and proved identical to the sequence of TP 1 purified with column 2.

The sequence of TP 2 was also determined using the liquid-pulse sequencer, but unfortunately no radiolabel was detected presumably because the total number of counts was insufficient. As expected from the purification, the sample contained two peptides, indicated by two initial sequences later decaying to a single sequence (Table 5.3). The longer peptide was 14 residues in length, whilst the shorter one was only 4 residues long and they presumably represented the main peak and shoulder respectively of Figure 5.16. All of the residues were identified except for residue 7 of the longer peptide which

presumably is the site of modification. Although the sequence of the tetrapeptide and the first 4 residues of the 14 residue peptide cannot be determined, residues 5 to 14 of the longer peptide represent additional sequence to that already determined.

The sequence of the 20 residue TP 3 was also determined (Table 5.4), and its hydrophobic nature indicated by its late elution from the reverse-phase column was confirmed as it contained a high proportion of hydrophobic residues such as alanine, leucine, phenylalanine, tryptophan and valine with a notably hydrophobic amino terminal half.

The tryptic peptide sequences were compared with the protein sequences in the University of Leeds OWL Composite Database (Release 6.0) (See Section 4.2.3). Although TP 1 and TP 2 (residues 5-14) were too short for a full database search, the TP 3 sequence was analysed but no significant homology with other proteins was apparent. Manual scanning of the TP 1 and TP 2 sequences did not produce any significant homology, even when the sequences were aligned with the active site regions of 8 homologous lactate and malate dehydrogenases, 10 residues either side of their catalytic histidines (Birktoft *et al.*, 1982), or with the sequence around the presumed active site histidine of cucumber NADH-dependent hydroxypyruvate reductase, *E. coli* D-3-phosphoglycerate dehydrogenase and the *E. coli pdx*B gene product; three proteins which have been proposed to form a family of 2-hydroxyacid dehydrogenases distinct from the lactate and malate dehydrogenase family (Grant, 1989). Furthermore, none of the tryptic peptides contained any known structural motifs as judged by comparison with a suite of structural motif matrices.

However, when the TP 1 sequence was aligned against the sequence of *P. putida* L(+)-mandelate dehydrogenase and mandelate racemase, a match of 4 out of the 7 residues was detected at the carboxy termini of both bacterial enzymes :

P. putida L(+)-mandelate dehydrogenase	T^{380} A P V D H L I G K G T H A^{393}-COO^-
R. graminis D(—)-mandelate dehydrogenase	V X L E I G K
P. putida mandelate racemase	I^{348} W R E K E I G K Y L V^{359}-COO^-

Finally, none of the tryptic peptide sequences was found within the amino terminal sequence of this enzyme (Section 4.2.3), and therefore the sequence of 94 residues, representing 27% of the polypeptide, has so far been determined.

Table 5.1 Amino acid sequence of TP 1

The sequence was determined by Dr D. Campbell, Department of Biochemistry, University of Dundee using an Applied Biosystems 470 A/120 A gas-phase sequencer equipped with on-line PTH-analysis. Approximately 0.2 nmole of pure peptide (400 cpm) was sequenced, and the PTH-amino acid identified at each cycle is given, along with the amount recovered in pmoles.

Cycle	PTH-amino acid	pmole
1	Val	53
2	Unk	—
3	Leu	65
4	Glu	24
5	Ile	64
6	Gly	24
7	Lys	64

Unk - Residue unknown

Table 5.2 Recovery of radiolabel and amino acid sequence of TP 1

The sequence was determined by Dr J. Keen at the SERC Sequencing Unit, Department of Biochemistry, University of Leeds using a solid-phase sequencer [Methods 2.15.6 (b)] and an Applied Biosystems 477-A liquid-pulse sequencer equipped with on-line PTH-analysis and scintillation counting. Approximately 0.2 nmole of peptide (400 cpm) was sequenced using the solid-phase sequencer, and approximately 1 nmole (2000 cpm) was sequenced using the liquid-pulse sequencer. The PTH-amino acid identified at each cycle is given, along with the amount recovered in pmoles.

Solid-phase sequencing

Cycle	PTH-amino acid	pmole	cpm above background
1	Unk	—	0
2	Unk	—	40
3	Leu	85	0
4	Glu	69	0
5	Ile	61	0
6	Gly	59	0

Liquid-pulse sequencing

Cycle	PTH-amino acid	pmole	cpm above background
1	Val	1060	0
2	Unk	—	105
3	Leu	925	94
4	Glu	575	114
5	Ile	767	131
6	Gly	723	41
7	Lys	496	0

Unk - Residue unkown

Table 5.3 Amino acid sequence of partially pure TP 2

The sequence was determined by Dr J. Keen at the SERC Sequencing Unit, Department of Biochemistry, University of Leeds using an Applied Biosystems 477-A liquid-pulse sequencer equipped with on-line PTH-analysis. The PTH-amino acid identified at each cycle is given along with the amount recovered in pmoles.

Cycle	PTH-amino acid	pmole	PTH-amino acid	pmole
1	Leu	20	Gly	12
2	Val	36	Ala	15
3	Val	48	Thr	20
4	Leu	49	Glu	10
5	Thr	30		
6	Thr	29		
7	Unk	—		
8	Ile	29		
9	Gly	29		
10	Gly	36		
11	Val	23		
12	Ala	26		
13	Ile	17		
14	Glu?	13		

Unk - Residue unknown. ? Identification tentative

Table 5.4 Amino acid sequence of TP 3

The sequence was determined by Dr J. Keen at the SERC Sequencing Unit, Department of Biochemistry, University of Leeds using an Applied Biosystems 477-A liquid-pulse sequencer equipped with on-line PTH-analysis. Approximately 1 nmole of peptide was sequenced, and the PTH-amino acid identified at each cycle is given, along with the amount recovered in pmoles.

Cycle	PTH-amino acid	pmole
1	Val	490
2	Phe	686
3	Ala	677
4	Ala	720
5	Ala	556
6	Gly	449
7	Ala	400
8	Gly	377
9	Phe	427
10	Asp	116
11	Trp	82
12	Leu	382
13	Asp	106
14	Leu	426
15	Asp	141
16	Ala	255
17	Leu	229
18	Asn	115
19	Glu	59
20	Arg	25

5.8 Analysis of modified amino acids by thin-layer electrophoresis

As sequence analysis had failed to determine the identity of the modified amino acid(s), thin-layer electrophoresis was used as a potential means of identification. D(—)-Mandelate dehydrogenase inactivated with D(—)-2-(bromo[^{14}C]acetyl)mandelate was acid hydrolysed for 24 h [Methods 2.15.3 (b)], a procedure which converts α-(halo ester)-modified amino acids to their carboxymethylated derivatives (Adamson *et al.*, 1984). After lyophilization, the hydrolysate was electrophoresed on thin-layer silica plates (Methods 2.15.5), and a typical plate is shown in Figure 5.17. Although more than one type of amino acid had been modified, as indicated by the presence of radiolabel in several fractions, the major peak co-migrated with the standard N^3-(carboxymethyl)histidine. This result suggests, but does not prove, that a histidine residue at the active site of D(—)-mandelate dehydrogenase might be the principal residue with which D(—)-2-(bromoacetyl)mandelate reacts.

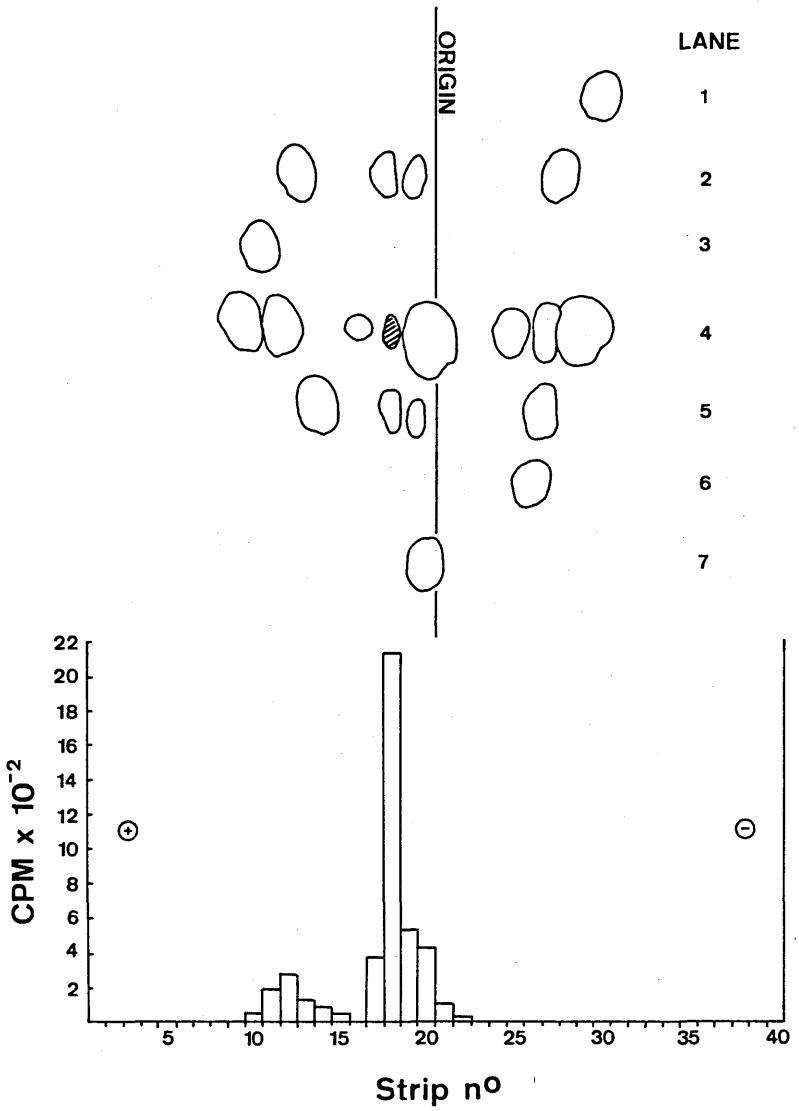


Figure 5.17 Thin-layer electrophoresis of an acid hydrolysate of D(—)-mandelate dehydrogenase inactivated with D(—)-2-(bromo[^{14}C]acetyl)mandelate

D(—)-Mandelate dehydrogenase (800 μg, 21 nmol) inactivated with D(—)-2-(bromo[^{14}C]acetyl)mandelate was acid hydrolysed for 24 h [Methods 2.15.3 (b)], lyophilized and a 4 nmol sample (5000 cpm) spotted onto a thin-layer silica plate along with neutral, basic and carboxymethylated amino acid standards. The plate was electrophoresed, dried, washed and the amino acids visualised as described in Methods 2.15.5. The plate was autoradiographed for 7 days (Methods 2.2.8), and then the lane containing the enzyme hydrolysate cut into 40 x 0.5 cm strips and each strip counted (Methods 2.2.8). The shaded area represents the most radioactive portion of the plate as determined by autoradiography.

Lane 1, 10 nmol arginine; lanes 2 & 5, 100 nmol mixture of (from right to left) histidine, N^1-(carboxymethyl)histidine, N^3-(carboxymethyl)histidine, N^1,N^3-(dicarboxymethyl)histidine; lane 3, 10 nmol *S*-carboxymethylcysteine; lane 4, 4 nmol D(—)-mandelate dehydrogenase hydrolysate; lane 6, 10 nmol histidine; lane 7, 13 nmol glycine.

5.9 Discussion

As described in Section 5.1, for a compound to be considered as an affinity label or active-site-directed inhibitor it must satisfy the following criteria (Meloche, 1967) : It should inhibit the enzyme completely and irreversibly, the inactivation should show rate saturation, the inactivation should be prevented by the substrate or by reversible inhibitors of the enzyme, and the binding of the inhibitor should be stoicheiometric. D(—)-2-(Bromoacetyl)mandelate satisfies all these criteria in its action upon D(—)-mandelate dehydrogenase from *R. graminis*, and it can therefore be classified as an affinity label of this enzyme.

The affinity of the enzyme for the inhibitor was clearly demonstrated by the protection afforded by D(—)-mandelate. A comparison of the apparent K_m for D(—)-mandelate at pH 8.0 (372 μM, Section 4.4.2) with the dissociation constant, K_i , for D(—)-2-(bromoacetyl)mandelate at the same pH (2.4 mM, Section 5.4) shows that although the enzyme binds the substrate more tightly, its affinity for the inhibitor is not too dissimilar, especially when considering that D(—)-2-(bromoacetyl)mandelate is notably larger than the natural substrate. Indeed, the relative stereochemistry of the affinity label, rather than its size, seems to be a more important determinant for binding, as the K_i for D(—)-2-(bromoacetyl)mandelate (2.4 mM) is lower than that of L(+)-mandelate (8.2 mM, Section 4.3.6), which has exactly the same functional groups as D(—)-mandelate but is of opposite configuration.

The importance of the groups for which the enzyme shows affinity, in orientating the bromoacetyl moeity for alkylation, is supported by the observation that the non-specific alkylating reagents, iodoacetic acid and iodoacetamide do not inhibit the enzyme (Section 4.3.8), and although they possess a similar electrophilic group to D(—)-2-(bromoacetyl)mandelate they presumably lack the structural features necessary for specific association at the mandelate binding site. This enhanced reactivity or "facilitation" (Plapp, 1982) is a common feature of affinity labels, and often a simple reagent possessing the reactive group but which lacks the affinity group(s) is either not inhibitory or is a much less potent inhibitor. For example, the importance of the carboxylate and phenyl groups of the affinity label α-phenylglycidate was demonstrated by the lack of inhibition or poorer inhibition of mandelate racemase by a series of related epoxide compounds. Glycidate, bearing a carboxylate group but no aromatic ring was not inhibitory whilst styrene oxide, bearing an aromatic ring but no carboxylate group was only partially inhibitory. However, *trans*-β-phenylglycidate and α-phenylglycidate which possess both carboxylate and phenyl groups showed marked inhibition. When mandelate racemase (0.5 mM) was incubated at 30°C in the presence of 3 mM-inhibitor, 25% of the original activity remained after 1 h with *trans*-β-phenylglycidate but in the presence of the same concentration of α-phenylglycidate the enzyme was completely inactive after only 30 min (Fee *et al.*, 1974*b*; Kenyon & Hegeman, 1977). Similarly, the affinity label 3-(bromoacetyl)chloramphenicol is a much more potent inhibitor of the *E. coli*

type III chloramphenicol acetyltransferase than is iodoacetamide, resulting in an enhancement of the apparent bimolecular rate constant to 6 orders of magnitude greater than with the non-specific reagent (Kleanthous *et al.*, 1985).

In all of the inactivation experiments reported in this chapter, D(—)-mandelate dehydrogenase was inactivated with D(—)-2-(bromoacetyl)mandelate in the absence of added NAD(H). This alone, suggests that the enzyme can bind its aromatic substrate in the absence of the cofactor, and therefore may use a different reaction mechanism to that of other NAD^+-dependent dehydrogenases such as lactate and malate dehydrogenase which obey ordered mechanisms with the cofactor binding first (Banaszak & Bradshaw, 1975; Holbrook *et al.*, 1975). However, as the last step in the purification of the enzyme involved elution from Matrex Gel Orange A with NADH, it is likely that the preparation of enzyme used for inactivation contained small amounts of the cofactor and thereby allowed the affinity label to bind. A similar phenomenon has been observed with the inactivation of *E. coli* isocitrate dehydrogenase with phenylglyoxal. The enzyme is protected against inactivation by $NADP^+$ or isocitrate, even though the reaction the enzyme catalyses is known to obey an ordered mechanism with the cofactor binding first (Nimmo, 1986; M^{c}Kee, 1989). This anomaly has been explained by the presence of small amounts of cofactor in the preparation after $NADP^+$-elution from the final column during purification (Nimmo & Craig, 1989), and this was later confirmed when the enzyme, derived from a recombinant over-expressing strain and purified without the use of cofactor elution, was found to be no longer protected by isocitrate (M^{c}Kee, 1989).

Substrate protection experiments in which D(—)-mandelate dehydrogenase was inactivated with D(—)-2-(bromo[^{14}C]acetyl)mandelate in the absence and presence of substrate, indicated that radiolabel was incorporated into two active site peptides. However, comparison of the TP 1 and TP 2 sequences shows that neither sequence is found within the other, indicating that two different regions at or near the active site have been modified. If one was a partial cleavage product of the other from trypsin digestion, a stretch of homology would be detected. As the enzyme is inactivated by D(—)-2-(bromo[^{14}C]acetyl)mandelate with a stoicheiometry of 1.27 moles of label bound per mole of subunit, the possibility exists that the minor contaminant also modifies the enzyme and may therefore be responsible for the incorporation of radiolabel at a second site. On the other hand, the dual labelling might be accounted for by D(—)-2-(bromo[^{14}C]acetyl)mandelate modifying both TP 1 and TP 2, either by the enzyme being able to bind the inhibitor in more than one orientation, thereby pointing the bromoacetyl side chain towards different reactive groups or, alternatively, it might bind the inhibitor in a single orientation but the flexibility of the bromoacetyl group [which can move up to several angstroms (Plapp, 1982)] and/or polypeptide chain, may put the reactive group within the reach of more than one amino acid side chain. Indeed, such a labelling pattern has been observed for the inactivation of pig kidney D-amino acid oxidase modified with 1-fluoro-2,4-dinitrobenzene. Peptide analysis of enzyme labelled with this reagent indicated that two active site residues, tyrosine-55 and lysine-204 found on

different peptides, were modified and although both modifications contributed to the overall loss of enzymic activity, the residues were labelled in a mutually exclusive manner (Swenson *et al.*, 1981). However, further work, especially inactivation of the enzyme with homogeneous inhibitor, is required to determine whether the labelling of two regions at or near the active site of D(—)-mandelate dehydrogenase is due to either labelling by the contaminant or dual labelling by D(—)-2-(bromo[^{14}C]acetyl)mandelate.

The detection of sequence similarity between TP 1 and residues at the carboxy termini of the *P. putida* mandelate racemase and L(+)-mandelate dehydrogenase (Section 5.7), raises the possibility that the yeast and bacterial enzymes may share other limited sequence similarity, as suggested by comparison of their amino acid compositions (Section 4.5.1). As TP 1 represents sequence at or near the active site of the *R. graminis* D(—)-mandelate dehydrogenase the residues might be involved in binding the substrate or promoting the necessary active site conformation and, moreover, as both mandelate racemase and L(+)-mandelate dehydrogenase also bind mandelate, the similar bacterial sequences could possibly represent residues which act in an analogous manner. Although crystal structures are not available for either of the mandelate dehydrogenases, the mandelate racemase crystal structure has recently been determined (G. A. Petsko, personal communication), and it will be of interest to see whether the carboxy terminal sequence of this enzyme forms part of the active site. Therefore, although the three enzymes are quite distinct proteins; a soluble, NAD^+-dependent dehydrogenase, a membrane-bound, flavin-dependent dehydrogenase and a metal-dependent racemase, which lack homology at their amino termini (Section 4.2.3) they may nevertheless share some limited sequence similarity dependent upon their common ability to bind mandelate i.e. they may have evolved common structure by a process of convergent evolution.

The inability to detect *S*-carboxymethylcysteine by amino acid analysis, peptide sequencing or electrophoresis of the modified amino acids, coupled with the marked insensitivity of the enzyme to a range of thiol reagents (Section 4.3.8) seems to rule out a direct role for the cysteine side chain in the catalytic mechanism of D(—)-mandelate dehydrogenase. Of the other likely nucleophiles that could participate in abstracting a proton from the hydroxyl group of D(—)-mandelate, histidine is an attractive candidate as the main peak of radiolabel, from a hydrolysate of inactivated enzyme, co-migrated with N^3-(carboxymethyl)histidine. Furthermore, other NAD^+-dependent 2-hydroxyacid dehydrogenases such as lactate and malate dehydrogenase are known to have an active site histidine residue which acts as an acid/base catalyst and, as part of a histidine-aspartate couple, participates in proton abstraction (Birktoft & Banaszak, 1983; Roderick & Banaszak, 1986; Clarke *et al.*, 1988). However, the lack of confirmatory evidence to support the identification of the modified residue as a histidine leaves this a matter for further investigation. The identity of the unknown residues of TP 1 and TP 2 will, ultimately, be determined by comparison of the peptide sequences with the complete amino acid sequence of D(—)-mandelate dehydrogenase.

CHAPTER 6

MANDELATE-UTILISING MICROORGANISMS AND IMMUNOLOGICAL RELATIONSHIPS AMONGST MANDELATE DEHYDROGENASES

6.1 Introduction

(a) Utilisation of mandelate by yeasts

Before the present study the only yeast to have been positively identified as being able to grow on D(—)- or L(+)-mandelate as its sole source of carbon and energy was *Rhodotorula graminis* strain KGX 39 (Durham *et al.*, 1984), although an unidentified yeast, isolated by selective enrichment, had also been reported to grow on L(+)-mandelate (Hegeman *et al.*, 1970). The ability of a number of yeasts and yeast-like fungi to utilise various aromatic compounds as their sole source of carbon had been the focus of several previous studies. However, they concentrated mainly on the metabolism of monophenolic compounds such as benzoate, catechol, phenol, and protocatechuate, and those compounds related to lignin and humic acid breakdown such as 4-hydroxybenzoate, coumarate, ferulate, syringaldehyde, vanillin and vanillate (Henderson, 1961*a*,*b*; Harris & Ricketts, 1962; Cain *et al.*, 1968; Mills *et al.*, 1971).

As mandelate had not previously been used as a test substrate, a limited survey was made of a number of yeasts from culture collections in order to determine whether other species were capable of growth on D,L-mandelate. In addition, attempts were made to isolate mandelate-utilising yeasts by selective enrichment from natural environments. Samples of plant origin, presumably rich in aromatic compounds, were used as the starting material for enrichment and included garden compost, silage, soil, rotting leaves and humus as well as the faeces from various herbivorous animals including cattle, deer, horse, rabbit, and sheep. These were inoculated into a selective medium in which D,L-mandelate was the only source of carbon, and once organisms had been identified as being able to grow on mandelate, crude extracts were prepared and assayed for mandelate dehydrogenase activity.

(b) Immunological comparison of mandelate dehydrogenases

In the absence of sequence data for the mandelate dehydrogenases of *A. calcoaceticus* and *L. curvatus* as well as the D(—)-2-hydroxyisocaproate dehydrogenase of *S. faecalis*, immunological techniques can be used as an indirect, but nevertheless reasonably reliable, method of indicating whether sequence homology exists between two or more proteins (Chalmers & Fewson, 1989*b*). An immunological comparison of the various mandelate dehydrogenases was therefore carried out using polyclonal antisera raised against two distinct forms of the enzyme; the soluble, NAD^+-dependent D(—)-mandelate dehydrogenase from *R. graminis* KGX 39 and the membrane-bound, FAD-dependent D(—)-mandelate dehydrogenase from *A. calcoaceticus* C1123. By incubating antisera with crude extracts of *A. calcoaceticus* C1219 [constitutive for D(—)- and L(+)-mandelate dehydrogenase], *R. graminis* KGX 39, *L. curvatus* and *S. faecalis* containing mandelate dehydrogenase, in their native forms and after having been denatured on Western blots, it was possible to determine whether any immunological relationship existed between the two groups of enzyme. Both native and denatured enzymes were tested since the antisera were polyclonal, and may therefore have contained antibodies specific for both continuous epitopes i.e. antigenic determinants comprised of peptide segments from the

linear amino acid sequence, and discontinuous epitopes i.e. antigenic determinants formed from segments distant in the primary structure but which are brought into close proximity by the folding of the polypeptide chain.

6.2 Enrichment and growth of mandelate-utilising yeasts

(a) Isolation of mandelate-utilising yeasts

In a preliminary experiment in which the chloramphenicol was omitted from the medium, the culture became rapidly overgrown with oval-shaped Gram negative bacteria, presumably species of *Acinetobacter*. Although microscopic observation of the culture showed that yeasts were growing and dividing, they were vastly outnumbered by the bacteria, and addition of chloramphenicol to inhibit bacterial growth proved essential for the isolation of yeasts.

Although 18 representative colonies were originally picked from the enrichment plates only 5 yeasts were subsequently found to grow on D,L-mandelate as their sole source of carbon and energy. Two of these (DB 2, isolated from rabbit faeces, and DB 3, isolated from garden compost) were indistinguishable from each other and were pink, budding yeasts growing on solid medium as smooth, shining, circular, raised colonies. A third pink yeast (DB 11, isolated from sheep faeces) also grew on solid medium with a similar colony morphology although it was distinguishable from DB 2 and DB 3 by its darker colour. The other two isolates (DB 10, isolated from sheep faeces and DB 18, isolated from deer faeces) were both white/cream in colour and grew on solid medium as smooth, shining, circular, raised colonies although the former had a less shiny appearance.

(b) Growth of yeasts on D,L-mandelate

All those yeasts tested for their ability to grow on D,L-mandelate as the sole source of carbon and energy are given in Table 6.1. Of the 34 strains tested, which represented at least 28 species and 17 different genera, only 11 yeasts could grow on D,L-mandelate. Of these, 6 were *bona fide* strains of *Rhodotorula* and although the other 5 were of unknown identity, 3 of these (DB 2, DB 3 and DB 11) were pink in colour and presumably members of this genus.

Table 6.1 Growth of yeasts on D,L-mandelate as the sole source of carbon and energy.

Cells previously grown on Sabouraud-dextrose agar were inoculated into 50 ml of yeast nitrogen base/mandelate medium (in 250 ml conical flasks) prepared as described in Methods 2.3.3 (b). The cultures were shaken (200 rev. min^{-1}) for 96 h at 23°C. Immediately after inoculation and at 24 h, 48 h, 72 h and 96 h, samples were aseptically removed and the OD_{595} determined as described in Methods 2.3.6.

Yeast	Growth after 24 h	48 h	72 h	96 h
DB 2	+++	+++*		
DB 3	+++	+++*		
DB 10	+	++	++*	
DB 11	+	+	++	++
DB 18	+	++	++	++*
Candida nitratophila NCYC 556	—	—	—	—
C. pseudotropicalis NCPF 3234	—	—	—	—
C. tropicalis NCYC 1503	—	—	—	—
C. utilis NCYC 321	—	—	—	—
Debaromyces hansenii CBS 767	—	—	—	—
Endomycopsis capularis CBS 2519	—	—	—	—
Hanseniaspora valbyensis CBS 479	—	—	—	—
Hansenula anomala NCYC 432	—	—	—	—
Lodderomyces elongisporus NCYC 928	—	—	—	—
Pachysolen tannophilus NCYC 614	—	—	—	—
Pichia membranaefaciens CBS 107	—	—	—	—
Rhodosporidium toruloides NCYC 819	—	—	—	—
Rhodotorula glutinis NCYC 59	+	++	++	++*
R. graminis KGX 39	++	++*		
R. graminis NCYC 502	—	—	—	—
R. graminis NCYC 980	+	+	+*	
R. graminis NCYC 1401	+	+	+*	
R. grinbergsii IJFM 6000	+	+	++	++
R. minuta NCYC 931	—	—	—	—
R. nothofagii CCY 20-19-1	—	—	—	—
R. piliminae NCYC 759	—	—	—	—
R. rubra NCYC 758	+	+	+	+
Saccharomyces bayanus CBS 380	—	—	—	—
Sporobolomyces roseus NCYC 1609	—	—	—	—
Torulopsis colliculosa CBS 133	—	—	—	—
Trichosporon cutaneum CBS 2466	—	—	—	—
Trigonopsis variabilis CBS 1040	—	—	—	—
Wickerhamia fluorescens CBS 4565	—	—	—	—
Wingia robertsii CBS 2934	—	—	—	—

— no growth detected

+ growth up to 5x the original OD_{595}

++ growth between 5x and 10x the original OD_{595}

+++ growth above 10x the original OD_{595}

* no increase in OD_{595} from the previous time point

6.3 Identification of mandelate dehydrogenase activities in yeast

The 11 yeasts identified as being able to grow on D,L-mandelate were grown in yeast nitrogen base/mandelate medium, harvested and crude extracts prepared for assay. The extracts were not only assayed for the NAD^+-dependent D(—)-mandelate dehydrogenase and dye-linked L(+)-mandelate dehydrogenase already established in *R. graminis* KGX 39 (Durham, 1984), but also for NAD^+-dependent L(+)-enantiomer specific activity, $NADP^+$-dependent D(—)- and L(+)-enantiomer specific activities as well as for dye-linked D(—)-enantiomer specific activity. In some cases the nicotinamide nucleotide-dependent enzymes were also assayed for their phenylglyoxylate reductase activity i.e. the reverse/reduction reaction, especially where the oxidation reaction was low or undetectable.

Mandelate dehydrogenase activity could be demonstrated in only 7 of the 11 yeasts which grew on D,L-mandelate as the sole source of carbon and energy. Those in which no activity could be measured were DB 10, DB 11 and DB 18 as well as *R. rubra* NCYC 758. All of the yeast extracts with mandelate dehydrogenase activity contained a dye-linked L(+)-mandelate dehydrogenase, and in *R. glutinis* NCYC 59 and *R. grinbergsii* IJFM 6000 this constituted the only activity detected (Table 6.2). NAD^+-dependent D(—)-mandelate dehydrogenase activity was found in *R. graminis* KGX 39, NCYC 980, DB2 and DB3 and could be measured by the oxidation of D(—)-mandelate with NAD^+, as well as by the reduction of phenylglyoxylate with NADH. However, in *R. graminis* NCYC 1401 only the reduction of phenylglyoxylate with NADH could be measured, presumably because of the low specific activity of the mandelate dehydrogenase which could be detected only using the more sensitive reverse assay (Section 4.3.1). In addition, a previously unreported NADPH-dependent phenylglyoxylate reductase activity was found in *R. graminis* NCYC 1401, DB2 and DB3 although it accounted for only 5.8%, 3.7% and 5.3% respectively of their NADH-dependent phenylglyoxylate reductase activities. As these enzymes were assayed in crude extract it is unclear whether this represents dual-specificity with respect to the cofactor used or whether it is an independent enzyme.

Table 6.2 Mandelate dehydrogenase activities of *Rhodotorula graminis* KGX 39, NCYC 980 and NCYC 1401, *R. glutinis* NCYC 59, *R. grinbergsii* IJFM 6000, and yeasts DB 2 and DB 3

Cells previously grown on Sabouraud-dextrose agar [Methods 2.3.5 (a)], were inoculated into 2 x 400 ml volumes of yeast nitrogen base/mandelate medium [Methods 2.3.3 (b)], and grown as described in Methods 2.3.5 (a). The cultures were then harvested (Methods 2.3.7) and crude extracts prepared as in Methods 2.9.1, except that the high speed centrifugation was omitted. The extraction buffer also contained the following protease inhibitors; benzamidine (15.6 mg l^{-1}), leupeptin (0.5 mg l^{-1}), pepstatin (0.68 mg l^{-1}), and PMSF (35 mg l^{-1}). The mandelate dehydrogenases were assayed as described in Methods 2.4.2 (b),(c) and 2.4.3. Values in parentheses represent the results from two independent experiments.

NAD^+-dependent D(—)-mandelate dehydrogenase activity

Yeast	units $(mg\ protein)^{-1}$
R. graminis KGX 39	0.44 (0.43, 0.44)
R. graminis NCYC 980	0.03 (0.03, 0.03)
DB 2	0.20 (0.19, 0.20)
DB 3	0.13 (0.12, 0.14)

NADH-dependent phenylglyoxylate reductase activity

Yeast	units $(mg\ protein)^{-1}$
R. graminis KGX 39	2.28 (2.12, 2.43)
R. graminis NCYC 980	1.09 (1.08, 1.09)
R. graminis NCYC 1401	0.17 (0.16, 0.17)
DB 2	1.09 (1.03, 1.14)
DB 3	0.76 (0.69, 0.83)

NADPH-dependent phenylglyoxylate reductase activity

Yeast	units $(mg\ protein)^{-1}$
R. graminis NCYC 1401	0.01 (0.01, 0.01)
DB 2	0.04 (0.04, 0.04)
DB 3	0.04 (0.04, 0.04)

dye-linked L(+)-mandelate dehydrogenase activity

Yeast	units $(mg\ protein)^{-1}$
R. graminis KGX 39	0.08 (0.08, 0.08)
R. graminis NCYC 980	0.03 (0.03, 0.03)
R. graminis NCYC 1401	0.01 (0.01, 0.009)
R. glutinis NCYC 59	0.07 (0.07, 0.07)
R. grinbergsii IJFM 6000	0.008 (0.007, 0.009)
DB 2	0.03 (0.03, 0.03)
DB 3	0.03 (0.03, 0.03)

6.4 Immunological cross-reactivity

6.4.1 Immuno-inhibition of activity

The ability of polyclonal antisera raised against the D(—)-mandelate dehydrogenase of *R. graminis* KGX 39 and the D(—)-mandelate dehydrogenase of *A. calcoaceticus* C1123 to inhibit the D(—)-mandelate dehydrogenase activity of *A. calcoaceticus* C1219, the D(—)- and L(+)-mandelate dehydrogenases of *R. graminis* KGX 39, the D(—)-mandelate dehydrogenase of *L. curvatus* and the D(—)-2-hydroxyisocaproate dehydrogenase of *S. faecalis* was investigated. The effect of the anti-*A. calcoaceticus* D(—)-mandelate dehydrogenase serum on the activity of the *A. calcoaceticus* L(+)-mandelate dehydrogenase was not tested as it had already been shown not to inhibit the enzyme (Fewson *et al.*, 1988). The various yeast and bacterial crude extracts were incubated in the presence of antiserum or rabbit normal serum, and the remaining enzymic activities in the supernatants, before and after centrifugation, were determined (Table 6.3). Of the various combinations of enzymes and antisera tested, the only positive cross-reactions, as indicated by inhibition of activity, were between the antisera and their homologous antigens to which they were raised. However, some of the enzymes gave higher activities after incubation with the antisera e.g. the D(—)-mandelate dehydrogenases of *A. calcoaceticus* and *L. curvatus* and the D(—)-2-hydroxyisocaproate dehydrogenase of *S. faecalis* (Table 6.3), although a similar effect was produced when the *A. calcoaceticus* L(+)-mandelate dehydrogenase was incubated with anti-*A. calcoaceticus* D(—)-mandelate dehydrogenase serum (Fewson *et al.*, 1988). This may therefore be a non-specific enhancement of activity dependent upon the protein concentration and, moreover, the *S. faecalis* D(—)-2-hydroxyisocaproate dehydrogenase is known to be stabilised in the presence of BSA (Yamazaki & Maeda, 1986).

Further inhibition studies were then carried out to determine if the D(—)-mandelate dehydrogenase of *R. graminis* KGX 39 was immunologically similar to any of the mandelate dehydrogenases identified in the other species of *Rhodotorula* and yeasts isolated from the wild. In a preliminary experiment, antiserum raised against the *R. graminis* KGX 39 D(—)-mandelate dehydrogenase (batch 253) inhibited the enzymic activity of its homologous antigen by 88%, and also inhibited the D(—)-mandelate dehydrogenases of *R. graminis* NCYC 980, DB 2 and DB 3 by 80%, 77% and 75% respectively. However, as for strain KGX 39, there was no inhibition of the dye-linked L(+)-mandelate dehydrogenases of DB 2 and DB 3. The positive cross-reactions were then quantified by immuno-titration with batches 253 and 319 of the antisera, as well as with rabbit normal serum as a control (Figure 6.1 & 6.2). The strongest cross-reactions were observed between the antisera and the D(—)-mandelate dehydrogenase of *R. graminis* KGX 39, the antigen to which it had been raised, although batch 253 had a higher titre than batch 319 as indicated by its ability to inhibit the enzyme at higher dilution. The patterns of inhibition of the *R. graminis* NCYC 980, DB 2 and DB 3 enzymes

were remarkably similar to each other, although the antisera showed less specificity towards them compared to the homologous cross-reactions, as indicated by the dilutions at which the enzymes were inhibited 50% :

	50% inhibition of activity	
	antiserum 253	antiserum 319
DB 2	1: 16 - 1: 32	1: 32 - 1: 64
DB 3	1: 16 - 1: 32	1: 16 - 1: 32
R. graminis NCYC 980	1: 16 - 1: 32	1: 8 - 1: 16
R. graminis KGX 39	1:256 - 1:512	1: 64 - 1:128

Table 6.3 Effect of antisera raised against the *R. graminis* KGX 39 D(—)-mandelate dehydrogenase and *A. calcoaceticus* C1123 D(—)-mandelate dehydrogenase on the activities of the *A. calcoaceticus* C1219 D(—)-mandelate dehydrogenase, *R. graminis* KGX 39 D(—)- and L(+)-mandelate dehydrogenases, *L. curvatus* D(—)-mandelate dehydrogenase and *S. faecalis* D(—)-2-hydroxyisocaproate dehydrogenase

Crude extract (100 μl) was mixed with antiserum or rabbit normal serum (100 μl) and the mixture stood on ice for 1 h. Remaining enzymic activity in the supernatant was then assayed before and after centrifugation as in Methods 2.11.2. Two samples of antisera, raised in separate rabbits against the *R. graminis* KGX 39 D(—)-mandelate dehydrogenase, as well as a single sample of antiserum raised against the *A. calcoaceticus* C1123 D(—)-mandelate dehydrogenase were used. Assays were done in duplicate and the mean value is given. The results are shown as the percentage of the enzymic rates obtained after incubation of the extracts with rabbit normal serum.

(a) anti-*R. graminis* D(—)-mandelate dehydrogenase

	Before centrifugation		After centrifugation	
	batch 253	batch 319	batch 253	batch 319
A. calcoaceticus D(—)-MDH	100	110	110	107
R. graminis D(—)-MDH	11	36	6	14
R. graminis L(+)-MDH	103	ND	ND	ND
L. curvatus D(—)-MDH	121	130	116	108
S. faecalis D(—)-2-HIDH	134	107	121	118

(b) anti-*A. calcoaceticus* D(—)-mandelate dehydrogenase

	Before centrifugation	After centrifugation
A. calcoaceticus D(—)-MDH	52	51
R. graminis D(—)-MDH	99	107
L. curvatus D(—)-MDH	113	113
S. faecalis D(—)-2-HIDH	109	138

ND, not determined

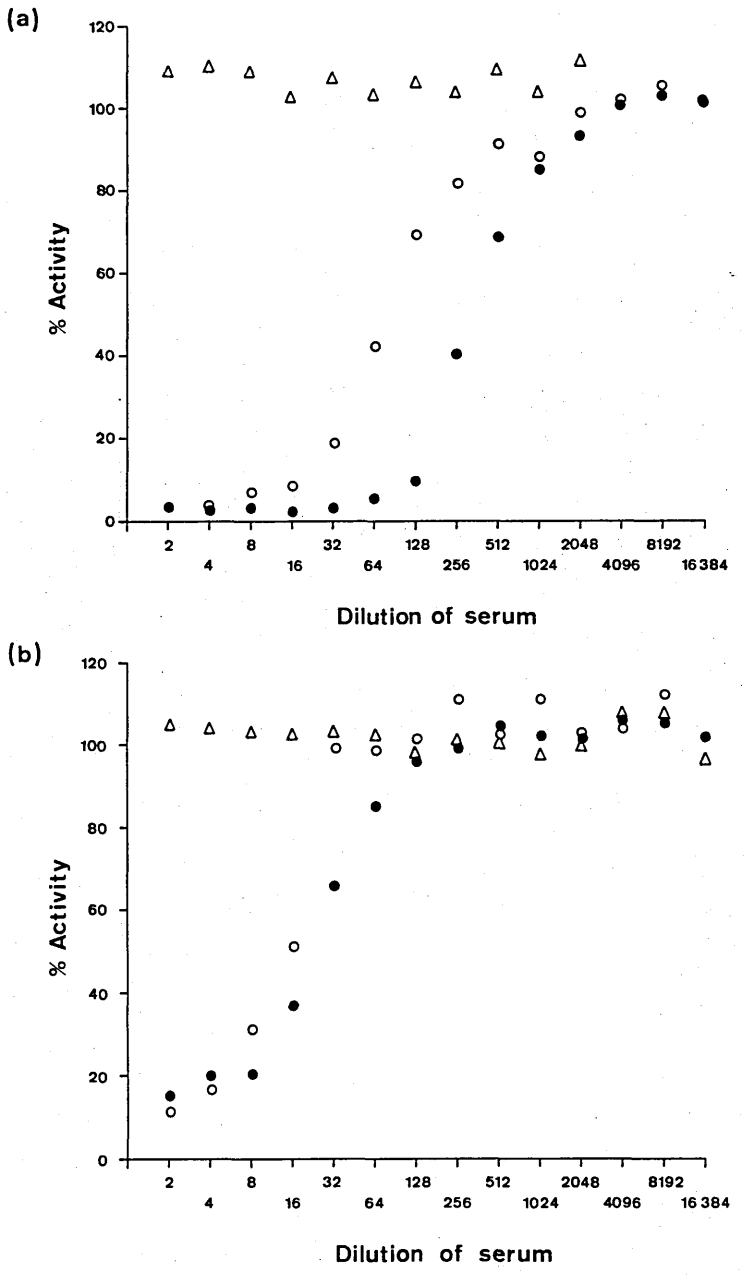


Figure 6.1 Immuno-titration of the D(—)-mandelate dehydrogenases of *R. graminis* KGX 39 and NCYC 980

Crude extracts were prepared as in Methods 2.9.1, except that high-speed centrifugation was omitted. The KGX 39 extract was diluted with 50 mM-Tris/HCl (pH 8.0) to give the same activity ml^{-1} as the NCYC 980 extract. Serial dilutions of antisera and rabbit normal serum [from undiluted serum to 1 : 8192 in 50 mM-Tris/HCl (pH 8.0)], were prepared and crude extract (200 μl) at the various dilutions and sera (200 μl), were mixed and stood on ice for 1 h. The remaining phenylglyoxylate reductase activity in the mixture was then assayed as in Methods 2.4.2 (c). Assays were carried out in duplicate and the results are expressed as the mean value as a percentage of the original 100% value.

(a) *R. graminis* KGX 39 D(—)-mandelate dehydrogenase, (b) *R. graminis* NCYC 980 D(—)-mandelate dehydrogenase. Antiserum batch 253 (●), antiserum batch 319 (○) and rabbit normal serum (△).

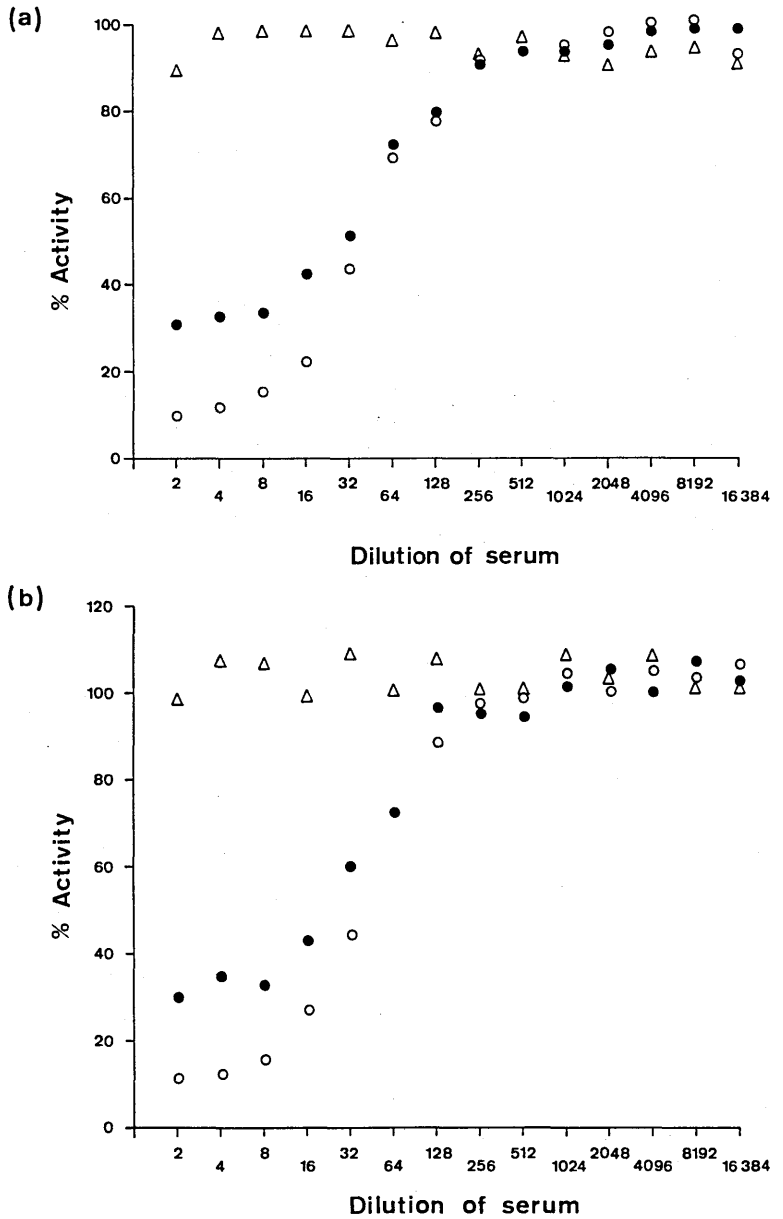


Figure 6.2 Immuno-titration of the D(—)-mandelate dehydrogenases of yeasts DB 2 and DB 3

Crude extracts were prepared as in Methods 2.9.1, except that high-speed centrifugation was omitted. Both extracts were diluted with 50 mM-Tris/HCl (pH 8.0) to give the same activity ml^{-1} as the *R. graminis* NCYC 980 extract (the lowest activity of the 4 extracts shown in Figure 6.1 and 6.2). Serial dilutions of antisera and rabbit normal serum [from undiluted serum to 1 : 8192 in 50 mM-Tris/HCl (pH 8.0)], were prepared and crude extract (200 μl) at the various dilutions and sera (200 μl), were mixed and stood on ice for 1 h. The remaining phenylglyoxylate reductase activity in the mixture was then assayed as in Methods 2.4.2 (c). Assays were carried out in duplicate and the results are expressed as the mean value as a percentage of the original 100% value.

(a) Yeast DB 2 D(—)-mandelate dehydrogenase, (b) yeast DB 3 D(—)-mandelate dehydrogenase. Antiserum batch 253 (●), antiserum batch 319 (○) and rabbit normal serum (△).

6.4.2 Immuno-blotting (Western blotting)

Immuno-blotting was used to determine whether any of the mandelate dehydrogenases would cross-react with the antisera, after being bound to nitrocellulose in the presence of SDS. Crude extracts of *A. calcoaceticus* C1219, *R. graminis* KGX 39, *L. curvatus* and *S. faecalis* were run on SDS-polyacrylamide gels and, after the proteins had been transferred to nitrocellulose, they were probed with the antiserum raised against the *A. calcoaceticus* C1123 D(—)-mandelate dehydrogenase as well as with antisera raised against the *R. graminis* KGX 39 D(—)-mandelate dehydrogenase (batches 253 and 319). Typical blots of the various extracts probed with the anti-*A. calcoaceticus* C1123 D(—)-mandelate dehydrogenase serum and anti-*R. graminis* KGX 39 D(—)-mandelate dehydrogenase serum are shown in Figures 6.3 and 6.4 respectively. The anti-*A. calcoaceticus* C1123 D(—)-mandelate dehydrogenase serum cross-reacted with several proteins in the *A. calcoaceticus* C1219 extract, although it reacted primarily with the D(—)-mandelate dehydrogenase band. However, it did not cross-react with the purified D(—)-mandelate dehydrogenase from *R. graminis* KGX 39 or with any other proteins in the various extracts tested (Figure 6.3). Likewise, the anti-*R. graminis* KGX 39 D(—)-mandelate dehydrogenase sera cross-reacted with the homologous antigen, in crude extract and purified form, and no other significant cross-reactions were evident (Figure 6.4). The blot shown in Figure 6.4 was probed with antiserum batch 253 although the same result was obtained when identical samples were probed with batch 319.

The positive cross-reactions between the anti-*R. graminis* KGX 39 D(—)-mandelate dehydrogenase sera and the native D(—)-mandelate dehydrogenases of *R. graminis* NCYC 980 and yeasts DB 2 and DB 3 were also analysed by immuno-blotting. In addition to extracts of mandelate-grown cells, equal loadings of extracts of glucose-grown cells were run as controls, as artifactual labelling of yeast proteins on nitrocellulose using polyclonal antisera has been reported to be a more common problem than with other organisms (Lillie & Brown, 1987). Therefore, by staining extracts of both induced and non-induced cells, any non-specific cross-reactions with proteins in the extracts of mandelate-grown cells should also be observed in the extracts of glucose-grown cells, with the exception of those with any other mandelate and β-ketoadipate pathway enzymes which are also induced during growth on mandelate (Durham *et al.*, 1984). Both batches of antisera cross-reacted with the homologous antigen in the purified form and in crude extract (Figure 6.5), confirming the pattern shown in Figure 6.4, lanes A & B. Moreover, the D(—)-mandelate dehydrogenase band in the crude extract (which had been prepared in the presence of protease inhibitors) co-migrated with the purified enzyme, indicating that detectable proteolytic cleavage had not occurred during the purification procedure (Sections 3.3-3.7). Both batches of antisera also cross-reacted with a single band of protein in the extracts prepared from mandelate-grown cells of yeasts DB 2 and DB 3, as did batch 253 with the extract of mandelate-grown *R. graminis* NCYC 980 (Figure 6.5). However, batch 319 cross-reacted with two bands of protein in the extract of mandelate-grown *R. graminis* NCYC 980

(Figure 6.5 (b), lane H), although the upper band was also stained in the extract of glucose-grown cells (Figure 6.5 (b), lane I). Comparison of the two staining patterns therefore enabled the lower of the two bands to be assigned as the specific cross-reaction and, moreover, it ran with the same relative mobility as the single band identified when the same extract was probed with batch 253 (Figure 6.5 (a), lane H). The strongest cross-reaction, as indicated by the intensity of staining for an equal loading of enzymic activity, was for the homologous antigen. The three other bands were less intense, but approximately equal with each other, and this is consistent with the inhibition patterns of the enzymes in their native forms (Section 6.4.1). The three single bands stained in the extracts of mandelate-grown DB 2, DB 3 and *R. graminis* NCYC 980 co-migrated on SDS-polyacrylamide gels, indicating a similar subunit size, although all three were slightly larger than the *R. graminis* KGX 39 D(—)-mandelate dehydrogenase subunit (Figure 6.5).

Staining of extracts prepared from glucose-grown cells also allowed identification of the mandelate dehydrogenases in the non-induced cultures. Direct observation of the blots showed faint bands in the *R. graminis* KGX 39, DB 2 and DB 3 extracts (but not in the *R. graminis* NCYC 980 extract), which migrated at the same position as the more heavily stained bands in the extracts of mandelate-grown cells. Unfortunately, they were of insufficient intensity to be visible on the photographs shown in Figure 6.5.

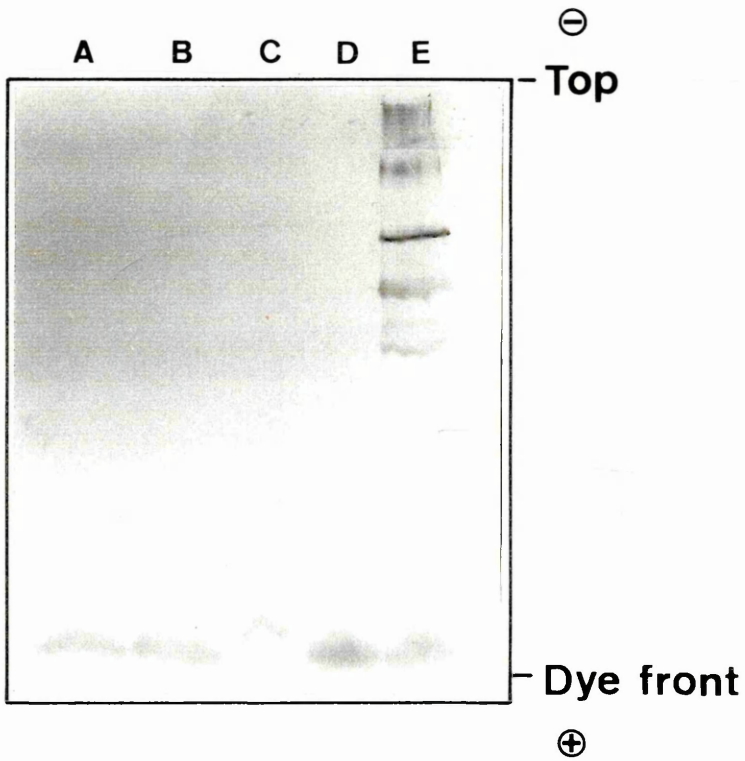


Figure 6.3 Immuno-blot of purified *R. graminis* KGX 39 D(—)-mandelate dehydrogenase and crude extracts of *R. graminis* KGX 39, *L. curvatus*, *S. faecalis* and *A. calcoaceticus* C1219, probed with antiserum raised against the D(—)-mandelate dehydrogenase from *A. calcoaceticus* C1123

Samples were electrophoresed in 12.5% (w/v) SDS-polyacrylamide gels as described in Methods 2.10.1. Proteins were then electroblotted onto nitrocellulose [Methods 2.11.3 (a)], and the blots developed as described in Methods 2.11.3 (b).

Lanes : A, 0.5 μg of purified *R. graminis* KGX 39 D(—)-mandelate dehydrogenase; B, 20 μg of *R. graminis* KGX 39 crude extract; C, 50 μg of *L. curvatus* crude extract; D, 50 μg of *S. faecalis* crude extract; E, 50 μg of *A. calcoaceticus* C1219 crude extract.

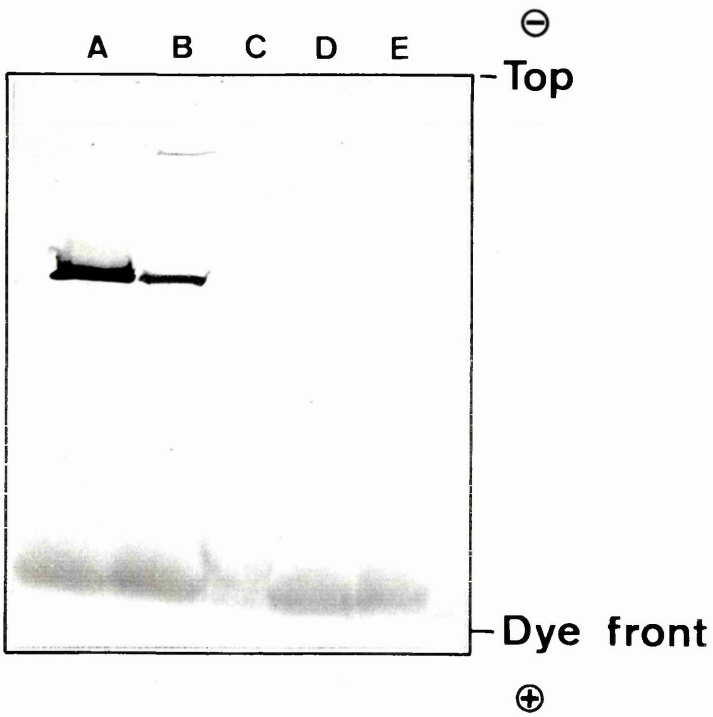


Figure 6.4 Immuno-blot of purified *R. graminis* KGX 39 D(—)-mandelate dehydrogenase and crude extracts of *R. graminis* KGX 39, *L. curvatus*, *S. faecalis* and *A. calcoaceticus* C1219, probed with antiserum raised against the D(—)-mandelate dehydrogenase from *R. graminis* KGX 39 (batch 253)

Samples were electrophoresed in 12.5% (w/v) SDS-polyacrylamide gels as described in Methods 2.10.1. Proteins were then electroblotted onto nitrocellulose [Methods 2.11.3 (a)] and the blots developed as described in Methods 2.11.3 (b).

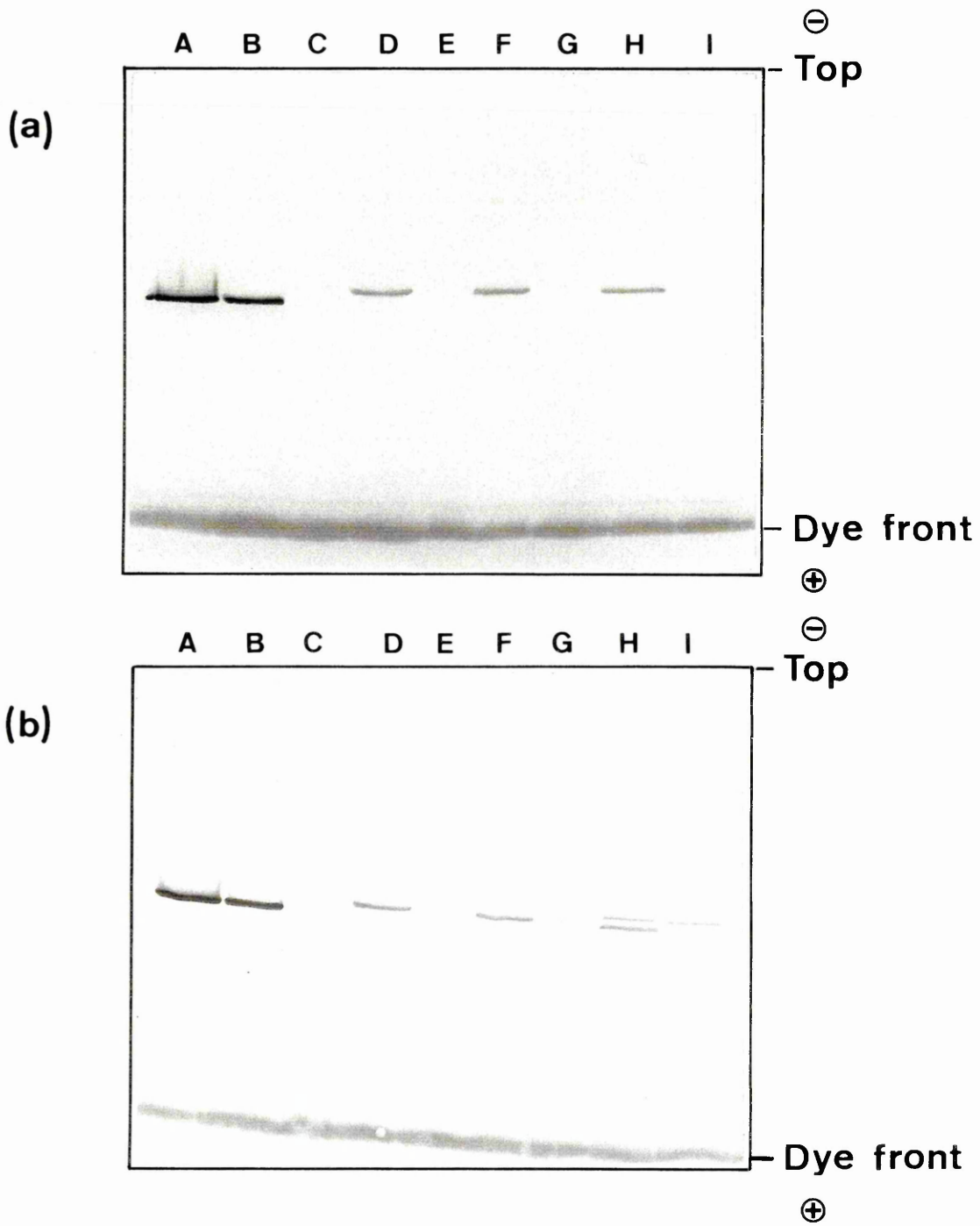
Lanes : A, 0.5 μg of purified *R. graminis* KGX 39 D(—)-mandelate dehydrogenase; B, 20 μg of *R. graminis* KGX 39 crude extract; C, 50 μg of *L. curvatus* crude extract; D, 50 μg of *S. faecalis* crude extract; E, 50 μg of *A. calcoaceticus* C1219 crude extract.

Figure 6.5 Immuno-blots of purified *R. graminis* KGX 39 D(—)-mandelate dehydrogenase and crude extracts of *R. graminis* KGX 39, NCYC 980 and yeasts DB 2 and DB 3, probed with antisera raised against the D(—)-mandelate dehydrogenase from *R. graminis* KGX 39

Crude extracts of glucose and mandelate-grown cells were prepared as in Methods 2.9.1, except that the high speed centrifiugation was omitted. The extraction buffer also contained the following protease inhibitors; benzamidine (15.6 mg l^{-1}), leupeptin (0.5 mg l^{-1}), pepstatin (0.68 mg l^{-1}), and PMSF (35 mg l^{-1}). Extracts of mandelate-grown cells with equal enzymic activities, (0.05 μmol min^{-1}), plus an equivalent amount of protein from extracts of glucose-grown cells were electrophoresed in 12.5% (w/v) SDS-polyacrylamide gels as in Methods 2.10.1. Proteins were then electroblotted onto nitrocellulose [Methods 2.11.3 (a)] and the blots developed as described in Methods 2.11.3 (b).

Lanes : A, 0.5 μg of purified *R. graminis* KGX 39 D(—)-mandelate dehydrogenase; B, 21 μg of mandelate-grown *R. graminis* KGX 39 crude extract; C, 21 μg of glucose-grown *R. graminis* KGX 39 crude extract; D, 41 μg of mandelate-grown DB 2 crude extract; E, 41 μg of glucose-grown DB 2 crude extract; F, 50 μg of mandelate-grown DB 3 crude extract; G, 50 μg of glucose-grown DB 3 crude extract; H, 50 μg of mandelate-grown *R. graminis* NCYC 980 crude extract; I, 50 μg of glucose-grown *R. graminis* NCYC 980 crude extract.

(a) Probed with batch 253, (b) probed with batch 319.


(a)
A B C D E F G H I
⊖
Top
Dye front
⊕
(b)
A B C D E F G H I
⊖
Top
Dye front
⊕

6.5 Discussion

Selective enrichment was successful in isolating several yeasts capable of growth on D,L-mandelate as their sole source of carbon and energy and, moreover, it was a quick and simple method of isolating organisms with the desired phenotype. In contrast, the identification and subsequent isolation of the D(—)-2-hydroxyisocaproate dehydrogenase from *S. faecalis*, an enzyme with marked D(—)-mandelate dehydrogenase activity, was based on screening crude extracts of 96 strains of bacteria and 93 strains of yeast and other fungi (Yamazaki & Maeda, 1986); a process which although successful, was nevertheless profligate of time, cost and effort.

Although the yeasts tested for their ability to grow on D,L-mandelate represented a number of genera, the experiment was not designed to include as comprehensive a range as possible. However, it is still noteworthy that nearly all of the isolates showing growth on mandelate were species of *Rhodotorula*, and this may therefore represent a common, although not universal, trait within the genus. Although *R. graminis* KGX 39, NCYC 980 and NCYC 1401 grew on D,L-mandelate, strain NCYC 502 did not, indicating that as with bacteria such as *A. calcoaceticus* and *P. putida*, the ability to grow on mandelate is a strain specific rather than a species specific property (Stanier *et al.*, 1966; Baumann *et al.*, 1968; Fewson, 1988*b*). Furthermore, not all of the strains of *Rhodotorula* possessed both D(—)-enantiomer specific and L(+)-enantiomer specific mandelate dehydrogenases (e.g. *R. glutinis* NCYC 59 and *R. grinbergsii* IJFM 6000), and presumably the faster growth rates and higher growth yields on D,L-mandelate of strains such as *R. graminis* KGX 39, DB 2 and DB 3, is reflected by the presence of both enzymes. The inability to detect mandelate dehydrogenase activity in *R. rubra* NCYC 758, DB 10, DB 11 and DB 18, even though they could grow on D,L-mandelate was presumably because the specific activities of the enzyme(s) were too low to be detected using the assays employed. Indeed, not all fungal mandelate dehydrogenases are as easy to assay as the enzymes of *R. graminis* KGX 39. The D(—)- and L(+)-mandelate dehydrogenases of *Aspergillus niger* strain UBC 814 have specific activities in crude extract of 0.81 munits (mg protein)$^{-1}$ and 0.45 munits (mg protein)$^{-1}$ respectively (Jamaluddin *et al.*, 1970), values which are 540 and 180 times less than the specific activities of the analogous enzymes in *R. graminis* KGX 39 (Table 6.2). Alternatively, the inability to detect mandelate dehydrogenase activity in these yeasts might indicate that oxidation by the appropriate dehydrogenase does not form the first step in the catabolism of mandelate in these organisms.

Although immunological studies have been used to demonstrate homology amongst analogous enzymes from different organisms, e.g. the protocatechuate 3,4-dioxygenases of *Azotobacter vinelandii*, *Pseudomonas aeruginosa* and *A. calcoaceticus*, and the benzaldehyde and benzyl alcohol dehydrogenases of *A. calcoaceticus* and *P. putida* (Durham *et al.*, 1980; Durham & Ornston, 1980;

Chalmers *et al.*, 1990*b*), no homology between the mandelate dehydrogenases of *R. graminis* KGX 39, *A. calcoaceticus* C1219, *L. curvatus* and the D(—)-2-hydroxyisocaproate dehydrogenase of *S. faecalis* was demonstrated.

In a systematic study of a number of avian lysozymes, Prager & Wilson (1971*a*) demonstrated a linear relationship between the sequence homology of the proteins and their immunological cross-reactivity. It was predicted that cross-reactivity would not be detectable above 40% sequence difference and subsequent analysis of a diverse range of other proteins generally confirmed this (Prager & Wilson, 1971*b*). Therefore, it is unlikely that any of the mandelate dehydrogenases listed above have more than 40% sequence identity with each other, and it is probable that values much less than this exist for pairwise comparisons between any of the soluble and membrane-bound enzymes, especially in view of the marked differences in properties and intracellular location. Moreover, if either of the antisera contained antibodies specific for epitope(s) at the mandelate binding site of the enzyme to which it was raised, the inability to inhibit the activity of the heterologous antigens in their native form suggests that although these sites have a common function i.e. the binding of mandelate, they do not share structural similarity. However, as pointed out by Arnheim *et al.* (1971) the "lack of cross-reactivity between two antigens using antisera directed against native structures does not necessarily indicate insignificant amino acid sequence similarity nor disparate three-dimensional structures". For example, antisera raised against the catabolic ornithine carbamoyltransferases of *Aeromonas formicans* and *P. aeruginosa*, cross-react strongly with the *E. coli* anabolic ornithine carbamoyltransferase (*arg*F product), but only weakly or not at all with the other *E. coli* anabolic ornithine carbamoyltransferase (*arg*I product), yet the two isoenzymes show an overall identity of 86% at the amino acid sequence level (Van Vliet *et al.*, 1984; Tricot *et al.*, 1989). Similarly, antisera raised against human leukaemia and hen egg-white lysozymes do not cross-react with their reciprocal, heterologous antigens even though the enzymes show 60% amino acid sequence identity and their three-dimensional structures are very similar (Arnheim *et al.*, 1971; Blake & Swann, 1971).

A method commonly used to overcome such anomalous behaviour is to denature the antigen prior to immunisation, by reduction and carboxymethylation of cysteine side chains, thereby exposing normally buried residues to the immune system of the animal in which the antiserum is raised. Although this technique has been successful in demonstrating heterologous cross-reactions which are not observed between antigens and antisera raised against their native forms (Arnheim *et al.*, 1971), it is unlikely that reduction and carboxymethylation of the single cysteine residue of the *R. graminis* KGX 39 D(—)-mandelate dehydrogenase would produce significant unfolding of the polypeptide, especially if it was located on the protein surface and exposed to solvent, rather than being buried. Indeed, proteins are often very tolerant of structural modification e.g. substitution of a number of alanine residues into the structure of human growth hormone resulted in mutant proteins in which the folding of the polypeptide chain was

indistinguishable from that of the wild-type hormone (Cunningham & Wells, 1989). Even though the various mandelate dehydrogenases were not probed with antisera raised against the denatured *A. calcoaceticus* C1123 and *R. graminis* KGX 39 D(—)-mandelate dehydrogenases, it is likely that both antisera contained antibodies specific for native and denatured forms, since they could inhibit the activity of their homologous antigens in solution and bind to them after denaturation with SDS, a phenomenon also observed with *A. calcoaceticus* and *P. putida* benzaldehyde and benzyl alcohol dehydrogenases probed with antisera raised against the native antigens (Chalmers *et al.*, 1990*b*).

In contrast, the NAD^+-dependent D(—)-mandelate dehydrogenases of the yeasts *R. graminis* KGX 39, NCYC 980, DB 2 and DB 3 are certainly related to each other, as indicated by the marked cross-reactions between the antisera and the heterologous antigens in both native and denatured forms (Figure 6.1, 6.2 & 6.5). Therefore, they are likely to have in excess of 60% sequence identity (Prager & Wilson, 1971*b*), although it is presumably less than 100% as the heterologous cross-reactions were noticeably weaker than the homologous ones. However, the dye-linked L(+)-mandelate dehydrogenases of these yeasts do not seem to be related immunologically to the NAD^+-dependent D(—)-mandelate dehydrogenases as cross-reactivity was not observed.

These experiments also highlighted a common problem associated with probing antigens with polyclonal antisera; that is the visualisation of non-specific cross-reactivity, which is particularly prevalent when testing crude extracts. For example, the extract of mandelate-grown *R. graminis* NCYC 980 probed with two independent batches of antisera, raised in different rabbits, resulted in the staining of two bands with one antiserum (batch 319) but only one with the other (batch 253). In this case, however, the inclusion of extracts of non-induced, glucose-grown cells as controls enabled the specific-cross reaction to be assigned, but it is clear that if only one antiserum had been used or if the non-induced culture had been omitted, incorrect conclusions may have been drawn. Indeed, variations amongst antisera from different rabbits and between different bleeds from the same rabbit are fairly common. For example, Lillie & Brown (1987) reported that a rabbit injected with a purified yeast protein p55 (M_r 55 000), produced immune-serum that stained a number of faint bands on a Western blot of yeast high-speed supernatant in addition to the expected one of M_r 55 000, whereas the pre-immune serum from this rabbit only stained the 55 000 band even though the rabbit had not been injected with the antigen.

Identification of trace amounts of D(—)-mandelate dehydrogenase in extracts of glucose-grown cells of *R. graminis* KGX 39, DB 2 and DB 3 by Western blotting, indicates that the enzymes are synthesized in the absence of the inducer and under conditions of catabolite repression. This is also supported by the observation that the specific activities of these enzymes in extracts of glucose-grown cells, as a percentage of the level in extracts of mandelate-grown cells, are 1.3%, 0.7%, and 0.7% respectively. Therefore, although the levels of these enzymes are regulated by mechanisms of induction and repression (Section 3.1; Durham, 1984), neither process is absolute and there is

presumably a continual but limited turnover of D(—)-mandelate dehydrogenase in these yeasts. If the inducer of D(—)-mandelate dehydrogenase is phenylglyoxylate, as is the case for the mandelate dehydrogenases of *A. calcoaceticus* and *P. putida* (Hegeman, 1966*b*,*c*; Livingstone & Fewson, 1972), the enzyme would presumably be able to convert any D(—)-mandelate entering the cell into phenylglyoxylate and so induce the expression of more enzyme. However, as the identity of the inducer of D(—)-mandelate dehydrogenase in these yeasts is as yet unknown it is unclear as to whether the presence of the enzymes under non-induced conditions has any physiological significance. The ability to detect D(—)-mandelate dehydrogenase activity in glucose-grown cells of *R. graminis* KGX 39 seems to contradict the results obtained by Durham (1984) and Middleton (1988), in which no activity was detected. However, in both these cases enzymic activity was assayed by following the oxidation of D(—)-mandelate, as compared to the above results where the reduction of phenylglyoxylate was measured. This would then account for the apparent discrepancy, as the forward/oxidation assay is notably less sensitive than the reverse/reduction assay (Section 4.3.1).

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

The successful purification and preliminary characterization of D(—)-mandelate dehydrogenase from the yeast *R. graminis* strain KGX 39 has allowed a comparison of its properties with other mandelate dehydrogenases, which had previously been purified only from bacteria, and with various NAD^+-dependent dehydrogenases.

The enzyme is analogous to both the D(—)-mandelate dehydrogenase of *L. curvatus* and the D(—)-2-hydroxyisocaproate dehydrogenase of *S. faecalis* (Yamazaki & Maeda, 1986; Hummel *et al.*, 1988). All three enzymes are dimers of identical subunits and have similar properties with respect to M_r, quaternary structure, cofactor specificity, reversibility of the reaction catalysed, pH dependence of activity and common 2-hydroxy and 2-oxocarboxylic acids that they bind.

In contrast, comparison of the *R. graminis* D(—)-mandelate dehydrogenase with the D(—)- and L(+)-mandelate dehydrogenases of *A. calcoaceticus* shows them to be very different. Both of these bacterial enzymes are flavin-dependent, integral membrane proteins that catalyse the irreversible oxidation of mandelate. They differ from the *R. graminis* D(—)-mandelate dehydrogenase with respect to intracellular location, M_r, quaternary structure, pH optimum, cofactor used and sensitivity to thiol reagents (Allison *et al.*, 1985*a*,*b*; Hoey *et al.*, 1987). Furthermore, neither of the bacterial enzymes is related immunologically to the yeast enzyme. The L(+)-mandelate dehydrogenase of *P. putida* is similar to the *A. calcoaceticus* L(+)-mandelate dehydrogenase and, likewise, differs from the *R. graminis* D(—)-mandelate dehydrogenase with respect to intracellular location, M_r, quaternary structure and cofactor used (Gunsalus *et al.*, 1953; Stanier *et al.*, 1953; Tsou *et al.*, 1990; C. A. Fewson, personal communication). The amino terminus of the *P. putida* L(+)-mandelate dehydrogenase also lacks homology with the *R. graminis* D(—)-mandelate dehydrogenase amino terminus and, even though there is some sequence similarity between TP 1 and the carboxy terminus of the *P. putida* enzyme, the two proteins are overall quite distinct. In addition to the bacterial $NAD(P)^+$-independent mandelate dehydrogenase, the *R. graminis* L(+)-mandelate dehydrogenase is also $NAD(P)^+$-independent, catalyses the irreversible oxidation of mandelate, presumably contains flavin and is membrane "associated" (Durham, 1984; M. Yasin, personal communication).

On the basis of these comparisons there seem to be two clearly definable groups of mandelate dehydrogenase in both prokaryotes and eukaryotes : integral membrane proteins requiring flavin as prosthetic group and soluble, NAD^+-dependent proteins. This dichotomy is similar to that for other enzymes such as fructosebisphosphate aldolase, fumarase, quinate dehydrogenase and 3-dehydroquinase which are also found in more than one form, although the differences between the two groups of mandelate dehydrogenase reflects more closely the differences between the NAD^+-dependent and $NAD(P)^+$-independent lactate dehydrogenases found in bacteria (Garvie, 1980).

However, classification of the various mandelate dehydrogenases into only two groups is perhaps rather superficial as regards their evolutionary origin, especially when one considers that marked differences occur between the enzymes within each group. For example, although all of the bacterial NAD(P)$^+$-independent enzymes are monomeric, integral membrane proteins requiring flavin as prosthetic group, the D(—)-mandelate dehydrogenase of *A. calcoaceticus* has a M_r of 60 000 and uses FAD, whereas the L(+)-mandelate dehydrogenases of *A. calcoaceticus* and *P. putida* have M_r values of 44 000 and use FMN. On the basis of these comparisons, the enzymes may be further divided into two sub-groups, one containing the *A. calcoaceticus* D(—)-mandelate dehydrogenase and the other containing the *A. calcoaceticus* and *P. putida* L(+)-mandelate dehydrogenases, with the two groups presumably having independent evolutionary origin. Indeed, this delineation is supported by the observation that the D(—)-mandelate dehydrogenase of *A. calcoaceticus* is more similar to the D(—)-lactate dehydrogenase of this bacterium, than it is to L(+)-mandelate dehydrogenase and, likewise, the L(+)-mandelate dehydrogenase is more similar to L(+)-lactate dehydrogenase than it is to D(—)-mandelate dehydrogenase (Table 7.1). Moreover, this has led to the suggestion that the pair of D(—)-enantiomer specific dehydrogenases had a common evolutionary origin, as did the pair of L(+)-enantiomer specific dehydrogenases but a common origin for all four enzymes is much less likely (Allison *et al.*, 1985*b*; Hoey *et al.*, 1987).

Similarly, the enzymes of the NAD$^+$-dependent group of mandelate dehydrogenases may be further divided into two sub-groups, one containing the *R. graminis* D(—)-mandelate dehydrogenase and the other containing the *L. curvatus* D(—)-mandelate dehydrogenase and *S. faecalis* D(—)-2-hydroxyisocaproate dehydrogenase. Although the three enzymes are similar in many respects (Table 7.2), there are significant differences between the *R. graminis* D(—)-mandelate dehydrogenase and the two bacterial enzymes. The yeast enzyme shows marked insensitivity to 4-(chloromercuri)benzoate, is unable to oxidise a number of aliphatic 2-hydroxyacids which are substrates of the *L. curvatus* and *S. faecalis* enzymes and, in addition, it is not related immunologically to either of the bacterial enzymes. Furthermore, inclusion of all three enzymes in the same group ignores the fact that whilst the *R. graminis* D(—)-mandelate dehydrogenase is an inducible enzyme which forms part of a metabolic pathway for the catabolism of D(—)-mandelate, the two bacterial enzymes are probably constitutively expressed and have a different physiological role. The two groups of NAD$^+$-dependent mandelate dehydrogenases are likely to have had independent evolutionary origin, and their ability to oxidise D(—)-mandelate and reduce phenylglyoxylate has presumably arisen by coincidence rather than by a common functional requirement. This is perhaps not too surprising when one considers that *R. graminis* is a nutritionally versatile yeast whereas *L. curvatus* and *S. faecalis* are nutritionally fastidious bacteria, and presumably the selection pressures to which they are subject are quite dissimilar (Deibel & Seeley, 1974; Rogosa, 1974; Fell *et al.*, 1984).

In conclusion, it seems highly unlikely that the *R. graminis* D(—)-mandelate dehydrogenase evolved from an $NAD(P)^+$-independent enzyme as represented by the D(—)- and L(+)-mandelate dehydrogenases of *A. calcoaceticus* and *P. putida*. It is also unlikely to have evolved by means of duplication and subsequent mutation of the gene encoding L(+)-mandelate dehydrogenase in this yeast, as not only enantiomer specificity but also cofactor dependence and therefore reaction mechanism would have required major modification.

A much more likely candidate for the D(—)-mandelate dehydrogenase ancestor is another soluble, NAD^+-dependent 2-hydroxyacid dehydrogenase. Recruitment of such an enzyme by mutation or duplication of an existing gene might have provided an enzyme with an established NAD^+-dependence and reaction mechanism capable of abstracting two hydrogens from a 2-hydroxyacid and, therefore, relatively few mutations at the substrate binding site might have been required to endow the resultant enzyme with D(—)-mandelate dehydrogenase activity. It is not too difficult to imagine such a process giving rise to D(—)-mandelate dehydrogenase activity, especially in view of the few mutations which were required at the active site of *Bacillus stearothermophilus* L(+)-lactate dehydrogenase in order to alter its substrate specificity away from oxidising lactate/reducing pyruvate towards oxidising malate/reducing oxaloacetate. In a rational re-design of the active site by site-directed mutagenesis, threonine-246 was mutated to a glycine thereby increasing the volume of the active site, glutamine-102 was mutated to an arginine to provide a counterion for the second carboxyl group of malate/oxaloacetate and aspartate-197 was mutated to an asparagine in order to avoid possible charge repulsion with the second carboxyl group. As a result, the triple-mutant enzyme catalysed the reduction of oxaloacetate (as determined by the ratio of k_{cat} / K_m) 500 times more effectively than the reduction of pyruvate, compared to the wild-type enzyme which catalysed the reduction of pyruvate 1000 times more effectively than the reduction of oxaloacetate and, furthermore, this change of substrate specificity was mirrored in the ability of the mutant enzyme to catalyse the oxidation of malate more effectively than the oxidation of L(+)-lactate (Clarke *et al.*, 1987). If D(—)-mandelate dehydrogenase had evolved from another 2-hydroxyacid dehydrogenase, then it is plausible that incorporation of hydrophobic residues, which could solvate the aromatic ring of D(—)-mandelate, might have been the only substitutions required to alter the substrate specificity of the ancestral enzyme.

Recruitment of the enzyme to the mandelate pathway of *R. graminis*, even though it functions against the equilibrium of the reaction and has relatively poor D(—)-mandelate dehydrogenase activity, presumably represented a strong selective advantage in enabling the yeast to grow on an additional carbon source. Indeed, recruitment of a reversible, NAD^+-dependent lactate dehydrogenase in the bacterium *Neisseria gonorrhoeae* seems to be the mechanism by which mutants of this bacterium overcome deficiency of the membrane-bound, $NAD(P)^+$-independent lactate dehydrogenase which normally catalyses the oxidation of lactate. The ability of the wild-type organism to grow on lactate,

phenyllactate or 4-hydroxyphenyllactate as the sole source of carbon and energy is endowed, in part, by a broad-specificity membrane-bound, $NAD(P)^+$-independent lactate dehydrogenase which irreversibly couples the oxidation of these 2-hydroxyacids to the reduction of *c*-type cytochromes (Bhatnagar *et al.*, 1989). However, mutants which lack this enzyme can still grow on lactate (but not on phenyllactate or 4-hydroxyphenyllactate), since they retain a cytoplasmic, NAD^+-dependent lactate dehydrogenase which presumably functions in the reverse-of-normal direction and, moreover, works against the equilibrium of the reaction in a similar manner to the *R. graminis* D(—)-mandelate dehydrogenase (Hendry *et al.*, 1990).

Finally, if the L(+)-mandelate dehydrogenase was already established in this yeast, which is possible in view of the fact that no strains of *Rhodotorula* were found to have a D(—)-mandelate dehydrogenase in the absence of an L(+)-mandelate dehydrogenase, it would have enabled the organism to utilise both enantiomers and therefore compete more favourably against other microorganisms.

Table 7.1 Comparison of the D(—)-mandelate and D(—)-lactate dehydrogenases and the L(+)-mandelate and L(+)-lactate dehydrogenases of *A. calcoaceticus*

Information is taken from Allison *et al.* (1985*a*,*b*), Allison & Fewson (1986) and Hoey *et al.* (1987). Reproduced from Hoey *et al.* (1987).

	L-Mandelate dehydrogenase	L-Lactate dehydrogenase	D-Mandelate dehydrogenase	D-Lactate dehydrogenase
Inducer	Phenylglyoxylate	D- or L-Lactate	Phenylglyoxylate	D- or L-Lactate
Location	All are integral components of the cytoplasmic membrane			
Solubilization by non-ionic detergents (e.g. Triton–X-100 or Lubrol) (% of activity released)	43–72	55–76	87–91	83–98
Solubilization by ionic detergents (e.g. cholate or deoxycholate) (% of activity released)	4–42	13–32	78–91	63–87
M_r (monomer)	44000	40000	60000	63000
pI value	4.2	< 4.0	5.5	5.8
pH optimum	7.5	7.5	8.0	7.7
Specific activity (units/mg of protein)	16.1	27.6	322	338
Stereospecificity	All are stereospecific for their substrate			
K_m value for substrate (μM)	186	83	385 (in addition D-lactate is a substrate but K_m = 4.8 mM)	308
Assay conditions	2,6-Dichloroindophenol reduction measured in the presence of *N*-methylphenazonium methosulphate or *N*-methylphenazonium ethosulphate. All require bovine serum albumin (or Triton X-100 in the case of D-mandelate dehydrogenase and D-lactate dehydrogenase) in the reaction mixture for full activity			
Inhibitors	None is affected by a wide range of chelating agents. All are moderately susceptible to several thiol-blocking reagents. All are inhibited by oxalate			
Inhibition by *p*-chloromercuribenzoate (concn. giving 50% inhibition)	Moderate (> 30 μM)	Moderate	Very severe (< 1 μM)	Severe (approx. 5 μM)
Cofactor (non-covalently bound)	FMN	FMN	FAD	FAD

Table 7.2 Comparison of the D(—)-mandelate dehydrogenase of *R. graminis*, the D(—)-mandelate dehydrogenase of *L. curvatus* and the D(—)-2-hydroxylsocaproate dehydrogenase of *S. faecalis*

Information is taken from this thesis, Yamazaki & Maeda (1986) and Hummel *et al.* (1988).

	R. graminis	*L. curvatus*	*S. faecalis*
Location	all are soluble enzymes		
Subunit M_r	38 000	30 000	34 000
Quaternary structure	all are dimeric enzymes		
pI value	5.9	ND	4.9
Cofactor	all use NAD(H) as a cofactor		
Reversibility of reaction catalysed	all catalyse reversible reactions		
pH optimum for D(—)-mandelate oxidation	9.5	8.5	9.2
pH optimum for phenylglyoxylate reduction	5.85	6.0	4.5
Apparent V value [units (mg protein)$^{-1}$] :			
D(—)-Mandelate oxidation	87 (pH 9.5)	ND	ND
Phenylglyoxylate reduction	572 (pH 5.85)	2122 (pH 6.0)	897 (pH 7.5)
K'_m for D(—)-mandelate (μM)	3050 (pH 7.0)	500 (pH 8.5)	ND
	325 (pH 9.5)		
K'_m for phenylglyoxylate (μM)	52 (pH 5.85)		
	46 (pH 7.0)	220 (pH 7.0)	3200 (pH 7.5)
K'_m for NAD^+ (μM)	309 (pH 7.0)		
	78 (pH 9.5)	210 (pH 8.5)	ND
K'_m for NADH (μM)	38 (pH 5.85)		
	36 (pH 7.0)	36 (pH 7.0)	35 (pH 7.5)
Inhibition by	None at	94% at	100% at
4-(chloromercuri)benzoate	5 mM	100 μM	500 μM

ND, Not determined

The primary aim of this study was to purify and characterize D(—)-mandelate dehydrogenase from *R. graminis* KGX 39 in order to compare it with other mandelate dehydrogenases and NAD^+-dependent dehydrogenases and, therefore, to allow speculation as to its evolutionary origin. However, the ability of the *R. graminis* D(—)-mandelate dehydrogenase to oxidise 4-hydroxy-3-methoxymandelate raises the possibility that this enzyme may be useful as a diagnostic test for determining the urinary concentration of this compound.

In mammals, including humans, D(—)-4-hydroxy-3-methoxymandelate (vanillymandelate) is a major breakdown product of the hormones adrenalin and noradrenalin, and is subsequently excreted from the body in the urine and faeces (Armstrong *et al.*, 1957; Smith *et al.*, 1959; Lun *et al.*, 1976). However, in pathological conditions such as neuroblastoma and certain hormonal diseases, the levels of urinary 4-hydroxy-3-methoxymandelate may be greatly elevated over the levels normally found in healthy individuals (Tuchman *et al.*, 1985). As a consequence, numerous methods for the quantitative determination of urinary 4-hydroxy-3-methoxymandelate have been developed, ranging from relatively simple techniques such as photometric analysis, paper chromatography, TLC and column chromatography, to more sophisticated ones such as gas chromatography, gas-liquid chromatography, HPLC and mass spectrometry (Pisano *et al.*, 1962; Godicke & Brosowski, 1964; Annino *et al.*, 1965; Messiha *et al.*, 1973; Lun *et al.*, 1976; Muskiet *et al.*, 1980; Tuchman *et al.*, 1983; Hitoshi *et al.*, 1989). However, several of these techniques are time consuming and lack specificity, and even those which are specific and quantitative are costly and often require complex laboratory equipment.

An alternative approach to these chemical techniques has been the use of specific enzymes. Although an early report claiming ability to measure urinary 4-hydroxy-3-methoxymandelate using an enzyme preparation of *P. fluorescens* strain A.3.12 was later invalidated by the absence of a D(—)-mandelate dehydrogenase in this organism, and the inability of its mandelate racemase to interconvert the D(—)- and L(+)-enantiomers of 4-hydroxy-3-methoxymandelate (Rosano, 1964; Weil-Malherbe, 1966), an enzymic method was subsequently developed using a strain of *P. putida* (Hegeman & Ellman, 1973). Despite this, enzymic methods do not seem to have been used in clinical practise.

However, with the discovery that purified D(—)-mandelate dehydrogenase from *R. graminis* KGX 39 and crude extracts of the yeasts DB 2 and DB 3 can oxidise 4-hydroxy-3-methoxymandelate, the possibility arises that these enzymes could be used for a cheap and sensitive diagnostic test for determining the urinary concentration of this compound. The sensitivity is reliant upon the substantial rate of change in A_{340} observed during the assay of the enzymes with D,L-4-hydroxy-3-methoxymandelate as substrate (Section 4.3.6), which accounts for 7.1, 5.9 and 5.6 times the rate of change in A_{340} obtained with D(—)-mandelate, using pure *R. graminis* KGX 39 D(—)-mandelate dehydrogenase and crude extracts of yeasts DB 2 and DB 3 respectively. Comparison of

the absorption spectra of assay mixtures at equilibrium, with D(—)-mandelate or D,L-4-hydroxy-3-methoxymandelate as substrate, have shown that whilst phenylglyoxylate has negligible absorbance at 340 nm i.e. the monitoring wavelength (Section 4.3.6), 4-hydroxy-3-methoxyphenylglyoxylate absorbs strongly at this wavelength and, moreover, produces a shift in the absorption maximum from 340 nm to 356 nm.

Before any of these enzymes could be used in such a diagnostic test, experiments would have to be carried out to determine the molar absorption coefficient of 4-hydroxy-3-methoxyphenylglyoxylate in order to assess its contribution to the total rate of change in A_{340} produced during assay. This could possibly be achieved by *de novo* synthesis, or by enzymically converting D(—)-4-hydroxy-3-methoxymandelate to its corresponding substituted phenylglyoxylate. However, as 4-hydroxy-3-methoxymandelic acid is commercially available only as a racemic mixture, then the D(—)-enantiomer would require separation from the L(+)-enantiomer prior to enzymic oxidation. In the course of the present work, attempts were made to separate the two enantiomers by chromatography on a chiral column, and although some success was achieved in producing partial separation, the two enantiomers could not be fully separated. If pure 4-hydroxy-3-methoxyphenylglyoxylic acid could be obtained, then the concentration of 4-hydroxy-3-methoxymandelate in solution could be determined from a standard curve of the final A_{340} at equilibrium and the ratio of the molar absorption coefficients at 340 nm of NADH and 4-hydroxy-3-methoxyphenylglyoxylate.

If this procedure was found to be suitable as a potential diagnostic test, clinical trials could then be carried out to determine the concentrations of 4-hydroxy-3-methoxymandelate in the urine of healthy individuals and patients with conditions producing abnormally high levels of this compound. Such a diagnostic test might involve spectrophotometric assay using the enzyme free in solution or, alternatively, the enzyme might be appropriate for use with a dip-stick if it could be coupled to a secondary enzyme e.g. an NADH-dependent reductase which could utilise the NADH produced by D(—)-mandelate dehydrogenase to reduce a suitable chromophore, and therefore indicate the concentration of 4-hydroxy-3-methoxymandelate by a colour change. It might also be necessary to over-express the enzyme in order to obtain large quantities, and the antisera raised against D(—)-mandelate dehydrogenase (Section 2.11.1) would therefore be useful for screening an expression library (Young & Davis, 1983).

Finally, if such a diagnostic test was successful it would represent a cheaper means of screening a larger number of individuals, and even if it were not as accurate as some of the chromatographic techniques available it might nevertheless identify those individuals for which further analysis was appropriate.

7.2 Future work

Although preliminary characterization of the *R. graminis* D(—)-mandelate dehydrogenase and comparison of its properties with other mandelate dehydrogenases and NAD^+-dependent dehydrogenases has allowed speculation as to its evolutionary origin, a more detailed study is required to further elucidate any evolutionary relationships this enzyme may have with analogous and possibly homologous enzymes. To determine whether any such relationships exist and, in particular, to answer those questions raised in this thesis, a number of potentially useful future experiments are outlined in the following sections.

7.2.1 Cloning and sequencing of the D(—)-mandelate dehydrogenase gene

Although approximately one quarter of the D(—)-mandelate dehydrogenase amino acid sequence has so far been determined (Section 4.2.3 & 5.7), a more detailed comparison with other enzymes cannot be made until the full sequence of the yeast protein is known. Therefore, the gene encoding D(—)-mandelate dehydrogenase should be cloned and sequenced, and this would not only give the nucleotide sequence but also allow the amino acid sequence to be deduced.

Although the genes of *S. cerevisiae* contain very few introns, thereby allowing cloning of genomic DNA which corresponds exactly to the amino acid sequences of the respective proteins, the coding regions of other fungal genes have been found to be punctuated with introns e.g. those of *Neurospora crassa*, *Schizosaccharomyces pombe* and *Trichoderma reesei* (Shoemaker *et al.*, 1983; Fink, 1987). Moreover, the phenylalanine ammonia lyase genes of *Rhodotorula rubra* and the related yeast *Rhodosporidium toruloides* contain 5 and 6 short introns respectively (Anson *et al.*, 1987; Vaslet *et al.*, 1988). Therefore, in order to avoid the possibility of cloning introns, the D(—)-mandelate dehydrogenase gene should be cloned from a cDNA library made from purified *R. graminis* mRNA. Then, after insertion into the appropriate vector(s) and transformation of *E. coli* or *S. cerevisiae*, positive clones should be identified using the oligonucleotide "probe" described in Section 4.5.1. The gene would then presumably be sequenced after cloning of restriction fragments into M13 sequencing vector(s).

Alternatively, in view of the fact that the subunit is only 350 or so residues in length, the sequence might more easily be determined by sequencing peptides produced from chemical and/or proteolytic cleavage. Indeed, cyanogen bromide cleavage as described by Kasper (1970) might be particularly useful in producing large peptides as the enzyme has relatively few methionine residues i.e. approximately 4 per subunit (Section 4.2.2).

Once the full gene and/or protein sequences are known, it would then enable a more thorough comparison with the archived sequences and structural motifs of other NAD^+-dependent dehydrogenases as well as with the sequences of the *P. putida* mandelate racemase and L(+)-mandelate dehydrogenase, although comparison of the

gene sequence would be more informative because of the degeneracy of the genetic code (Yokoyama *et al.*, 1990). In addition, sequencing would enable the identity of the unknown residues of tryptic peptides 1 and 2 to be determined.

7.2.2 Further active site studies

Although various active site studies have already been carried out on the *R. graminis* D(—)-mandelate dehydrogenase, comparatively little is known about the active site of this enzyme as compared to the wealth of information available for other NAD^+-dependent dehydrogenases. Therefore, a number of additional experiments are proposed which may be useful in identifying residues at the active site. Firstly, inhibition studies using diethylpyrocarbonate might indicate whether there is an active site histidine, which although it was suggested from the analysis of acid hydrolysates of enzyme inactivated with D(—)-2-(bromo[^{14}C]acetyl)mandelate (Section 5.8), was nevertheless not proven. In addition, the reagent phenylglyoxal could also be tried as a potential inhibitor as it is structurally analogous to phenylglyoxylate and, as it is considered as an arginine specific reagent, might indicate the presence of an active site arginine residue which could potentially form a salt bridge with the carboxylate group of D(—)-mandelate and phenylglyoxylate.

It would also be of importance to obtain homogeneous D(—)-2-(bromoacetyl)mandelic acid to repeat the inactivation studies and, in particular, the stoicheiometry of inactivation with affinity label free of contaminant. Furthermore, inactivation of D(—)-mandelate dehydrogenase at various values of pH should enable the pK_a values of the groups which bind and react with the affinity label to be determined, and comparison with the pK_a values obtained from the effect of pH on k_{cat}/K_m, should indicate whether the same groups are involved in substrate binding and catalysis (Fersht, 1985).

7.2.3 Determination of the 3-dimensional structure of D(—)-mandelate dehydrogenase by X-ray crystallography

Ultimately, the crystal structure of D(—)-mandelate dehydrogenase should be determined in order to allow comparison with a number of other NAD^+-dependent dehydrogenases for which crystal structures are available. In addition, comparisons could also be made with other mandelate pathway enzymes for which crystal structures are available or are presently being sought i.e. mandelate racemase and phenylglyoxylate decarboxylase from *P. putida* (Neidhart *et al.*, 1988; G. A. Petsko, personal communication). The ability to purify tens of milligrams of homogeneous D(—)-mandelate dehydrogenase of sufficient purity for crystallographic study, without the prior need to over-express the protein should be of substantial benefit although, if the enzyme does crystallise, its sequence would have to be determined in order to fit the amino acid sequence to its electron density map.

If the crystal structure of D(—)-mandelate dehydrogenase was determined it would allow some important questions regarding the structural evolution of this enzyme to be answered. For example, is the subunit composed of two distinct domains and, if so, does one of the domains bind the NAD^+ and have the characteristic nucleotide-binding fold found in other NAD^+-dependent dehydrogenases such as lactate, malate, alcohol and glyceraldehyde-3-phosphate dehydrogenases ? If so, it would support the notion that D(—)-mandelate dehydrogenase has evolved by recruitment of an NAD^+-dependent dehydrogenase from another metabolic pathway.

Analysis of the crystal structure might also indicate which residues at the substrate binding site are responsible for the enzyme's apparent specificity towards ring-containing substrates. In this respect, comparison with the crystal structures of mandelate racemase and phenylglyoxylate decarboxylase would be particularly interesting as D(—)-mandelate dehydrogenase binds the substrates of both these enzymes. Determination of the crystal structure, along with further active site studies and kinetic analysis should enable the role of specific active site residues in substrate binding and catalysis to be determined.

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