Mechanistic and active-site studies on D(–)-mandelate dehydrogenase from *Rhodotorula graminis*

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D(–)-Mandelate dehydrogenase, the first enzyme of the mandelate pathway in the yeast *Rhodotorula graminis*, catalyses the NAD⁺-dependent oxidation of D(–)-mandelate to phenylglyoxylate. D(–)-2-(Bromoethanoyloxy)-2-phenylethanoic acid ['D(–)-bromoacetylmandelic acid'], an analogue of the natural substrate, was synthesized as a probe for reactive and accessible nucleophilic groups within the active site of the enzyme. D(–)-Mandelate dehydrogenase was inactivated by D(–)-bromoacetylmandelate in a pseudo-first-order process. D(–)-Mandelate protected against inactivation, suggesting that the residue that reacts with the inhibitor is located at or near the active site. Complete inactivation of the enzyme resulted in the incorporation of approx. 1 mol of label/mol of enzyme subunit. D(–)-Mandelate dehydrogenase that had been inactivated with ¹⁴C-labelled D(–)-bromoacetylmandelate was digested with trypsin; there was substantial incorporation of ¹⁴C into two tryptic-digest peptides, and this was lowered in the presence of substrate. One of the tryptic peptides had the sequence Val-Xaa-Leu-Glu-Ile-Gly-Lys, with the residue at the second position being the site of radiolabel incorporation. The complete sequence of the second peptide was not determined, but it was probably an *N*-terminally extended version of the first peptide. High-voltage electrophoresis of the products of hydrolysis of modified protein showed that the major peak of radioactivity co-migrated with *N*-carboxymethylhistidine, indicating that a histidine residue at the active site of the enzyme is the most likely nucleophile with which D(–)-bromoacetylmandelate reacts. D(–)-Mandelate dehydrogenase was incubated with phenylglyoxylate and either (4*S*)-[4-³H]NADH or (4*R*)-[4-³H]NADH and then the resulting D(–)-mandelate and NAD⁺ were isolated. The enzyme transferred the *pro-R*-hydrogen atom from NADH during the reduction of phenylglyoxylate. The results are discussed with particular reference to the possibility that this enzyme evolved by the recruitment of a 2-hydroxy acid dehydrogenase from another metabolic pathway.

INTRODUCTION

The enzymes of the mandelate pathway enable various micro-organisms to grow on D(–), L(+) or DL-mandelate as the sole source of carbon and energy. In several bacteria, including *Acinetobacter calcoaceticus*, mandelate is degraded to benzoate by the successive action of either D(–) or L(+)–mandelate dehydrogenase, phenylglyoxylate decarboxylase and benzaldehyde dehydrogenase, although in certain strains of *Pseudomonas putida* D(–)-mandelate is first converted into phenylglyoxylate by mandelate racemase and L(+)–mandelate dehydrogenase (Fewson, 1988). The mandelate pathway has served as a model system for the study of peripheral metabolic pathways, and recent sequence analysis and crystallographic studies of the *Pseudomonas* enzymes have illuminated the mechanisms by which the pathway may have evolved in bacteria by the recruitment of enzymes from other metabolic pathways (Ransom *et al.*, 1988; Tsou *et al.*, 1990; Neidhart *et al.*, 1990). By contrast, little is known about the enzymes of the mandelate pathway in fungi, although we have previously reported the purification and preliminary characterization of an NAD⁺-dependent D(–)-mandelate dehydrogenase from the yeast *Rhodotorula graminis* (Baker & Fewson, 1989). This enzyme is quite unlike the membrane-bound flavin-dependent mandelate dehydrogenases of either *A. calcoaceticus* or *Ps. putida*, and therefore clearly belongs to a different class of enzyme (Baker &

Fewson, 1989; Baker, 1990). As part of our wider study of the evolution of the mandelate pathway in prokaryotes and eukaryotes, this novel mandelate dehydrogenase was chosen for further mechanistic and active-site studies. *In vitro* the enzyme catalyses the reversible interconversion of D(–)-mandelate and phenylglyoxylate, and is therefore analogous to the NAD⁺-dependent lactate dehydrogenases, which catalyse the reversible interconversion of lactate and pyruvate (Holbrook *et al.*, 1975). During the reduction of pyruvate by lactate dehydrogenase the two hydrogen atoms are donated to pyruvate in the form of a proton from the protonated imidazole group of an active-site histidine residue and a hydride ion from the 4-position of the dihydronicotinamide ring of NADH (Clarke *et al.*, 1989). Since D(–)-mandelate dehydrogenase from *R. graminis* is also an NAD⁺-dependent enzyme, we wished to determine which active-site residue(s) are involved in catalysis and which of the two hydrogen atoms at the 4-position of the dihydronicotinamide ring of NADH is transferred to phenylglyoxylate. In order to probe the active site for accessible nucleophilic groups the compound D(–)-2-(bromoethanoyloxy)-2-phenylethanoic acid [which we refer to as 'D(–)-bromoacetylmandelic acid'] was chosen as a potential affinity label of the enzyme since it retains both the phenyl and carboxylate groups found in the natural substrate, but also includes a reactive electrophilic bromoacetyl group (Fig. 1).

The present paper describes the synthesis, purification and

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Fig. 1. Structures of D(-)-mandelic acid and D(-)-2-(bromoethanoyloxy)-2-phenylethanoic acid [D(-)-bromoacetylmandelic acid]

characterization of D(-)-bromoacetylmandelic acid, which was then used to inactivate D(-)-mandelate dehydrogenase. An active-site peptide was isolated and characterized and the stereospecificity of hydride transfer of the enzyme was determined. The paper concludes with a discussion of the possible origin and evolution of this enzyme.

EXPERIMENTAL

Materials

Anhydrous acetonitrile, anhydrous pyridine, bromoacetic acid, bromoacetyl bromide and isobutyl chloroformate were from Aldrich Chemical Co., Gillingham, Dorset, U.K. Bromo[1-¹⁴C]acetic acid and D-[1-³H]glucose were from Amersham International, Little Chalfont, Bucks., U.K. Acetonitrile (h.p.l.c. grade) and *Saccharomyces cerevisiae* alcohol dehydrogenase were from BDH Chemicals, Poole, Dorset, U.K. Dichloromethane and 4-methylmorpholine were from Rathburn Chemicals, Walkersburn, Borders, U.K. *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase and *S. cerevisiae* hexokinase were from Sigma Chemical Co., Poole, Dorset, U.K. Carboxymethylated derivatives of histidine were synthesized as described by Kleantous *et al.* (1985) and identified by their relative mobilities after high-voltage electrophoresis (Adamson *et al.*, 1984). D(-)-Mandelate dehydrogenase was purified from *R. graminis* according to the method of Baker & Fewson (1989).

Synthesis of D(-)-bromoacetylmandelic acid

D(-)-Mandelic acid (1 g, 6.5 mmol) was dissolved in anhydrous acetonitrile (10 ml) at 0 °C and stirred under N₂. Bromoacetyl bromide (578 µl, 6.5 mmol) was added, followed by anhydrous pyridine (531 µl, 6.5 mmol), and the mixture was stirred for 30 min at 0 °C. It was then stirred for a further 30 min at room temperature. The reaction was monitored by t.l.c. on silica-gel plates, with toluene/ethyl acetate/formic acid (5:1:1, by vol.) as solvent. The synthesis yielded two products, D(-)-bromoacetylmandelic acid, which migrated with an *R_f* value of 0.43, and an unknown minor contaminant, with an *R_f* value of 0.50. The two products were separated from pyridine and unchanged D(-)-mandelic acid by 'flash' chromatography (Still *et al.*, 1978) on a column (23.5 cm × 2.5 cm) of Silica Gel 60 (Fluka Chemicals, Buchs, Switzerland) eluted with toluene/ethyl acetate (5:1, v/v) containing 8.3 % (v/v) formic acid. Fractions containing D(-)-bromoacetylmandelic acid were identified by t.l.c. and evaporated. Attempts were made to improve the separation of the two products by using other solvent systems, but none resulted in a better separation than the one described above. Attempts at crystallizing D(-)-bromoacetylmandelic acid from several single and double solvent systems were also unsuccessful, as was preparative t.l.c., because the compound was unstable under the conditions used.

M.s. yielded a pair of parent molecular ions with *m/z* values of 272 and 274, consistent with C₁₀H₉BrO₄. ¹H n.m.r.: δ (p.p.m.) ([²H]chloroform) 3.96 (2H, s), 6.00 (1H, s), 7.39–7.52 (5H, m)

and 12.01 (1H, s). There was also a resonance at 3.85 p.p.m. (s), which presumably represented a signal from the contaminant. Found: C, 42.6; H, 2.7; Br, 30.4; O, not determined; C₁₀H₉BrO₄ requires: C, 44.1; H, 3.3; Br, 29.0; O, 23.5 %. The c.d. spectrum (200–250 nm) of D(-)-bromoacetylmandelic acid was almost identical with that of authentic D(-)-mandelic acid, and both gave negative Cotton effects with maxima at 220 nm and 223 nm respectively, whereas authentic L(+)-mandelic acid gave the mirror image of D(-)-mandelic acid.

Synthesis of ¹⁴C-labelled D(-)-bromoacetylmandelic acid

Bromo[1-¹⁴C]acetic acid (5 µmol; 56 µCi/µmol) and bromoacetic acid (34 mg, 245 µmol) were dissolved in dichloromethane (12 ml) and cooled to -20 °C. 4-Methylmorpholine (27.8 µl, 250 µmol) was added, followed by isobutyl chloroformate (32.8 µl, 250 µmol), and the mixture was stirred for 2 min. Then D(-)-mandelic acid (38 mg, 250 µmol) dissolved in dichloromethane (50 ml) at -20 °C was added and the mixture was stirred for 4 h at -20 °C, followed by an overnight incubation at -20 °C without further stirring. The resulting ¹⁴C-labelled D(-)-bromoacetylmandelic acid {2-(bromo[¹⁴C₁]-ethanoyloxy)-2-phenylethanoic acid} (specific radioactivity 1.12 nCi/nmol) was purified as described above. The purified ¹⁴C-labelled D(-)-bromoacetylmandelic acid contained the same minor contaminant, as judged by its *R_f* value on t.l.c., that appeared with the first method of synthesis. After autoradiography of the t.l.c. plate, both products were found to be radioactive, although the amount of radioactivity incorporated into the contaminant was only about 9 % of that incorporated into ¹⁴C-labelled D(-)-bromoacetylmandelic acid.

Measurement of D(-)-mandelate dehydrogenase activity

D(-)-Mandelate dehydrogenase catalyses the oxidation of D(-)-mandelate by NAD⁺ and the reduction of phenylglyoxylate by NADH (Baker & Fewson, 1989). In the present work phenylglyoxylate reductase activity was measured in a 1 ml reaction mixture containing 200 mM-potassium phosphate buffer, pH 5.85, 200 µM-NADH and 1 mM-phenylglyoxylate as described previously (Baker & Fewson, 1989).

Inactivation of D(-)-mandelate dehydrogenase by D(-)-bromoacetylmandelate in the presence and in the absence of D(-)-mandelate

The time course of inactivation was measured at pH 8.0 in 50 mM-Tris/HCl buffer at 27 °C. The inactivation mixture (100 µl) contained D(-)-mandelate dehydrogenase (19 µg, 5 µM), D(-)-bromoacetylmandelate (1–8 mM) and acetonitrile (0.95 M). Experiments designed to test for protection also contained 5 mM-D(-)-mandelate. Immediately after addition of the label, the inactivation mixture was vortex-mixed and a sample (10 µl) was removed and diluted into 990 µl of 50 mM-Tris/HCl buffer, pH 8.0. Samples (10 µl) were then assayed for phenylglyoxylate reductase activity. At intervals throughout the inactivation, similar 10 µl volumes were removed and diluted, and appropriate samples (10–40 µl) were 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 with mixtures containing enzyme and acetonitrile but no inhibitor were carried out in parallel.

Incorporation of label after inactivation of D(-)-mandelate dehydrogenase by ¹⁴C-labelled D(-)-bromoacetylmandelate

The inactivation mixture (1125 µl) contained 50 mM-Tris/HCl buffer, pH 8.0, D(-)-mandelate dehydrogenase (1.3 mg, 30.4 µM), ¹⁴C-labelled D(-)-bromoacetylmandelate (1.4 mM) and acetonitrile (420 mM). Immediately after addition of the label,

the inactivation mixture was vortex-mixed and a sample (5 μ l) was removed and diluted into 995 μ l of 50 mM-Tris/HCl buffer, pH 8.0. This was further diluted (250 μ l into 750 μ l of the same buffer) before samples (10 μ l) were assayed for enzymic activity. At intervals throughout the inactivation, similar 5 μ l volumes were removed and diluted, and appropriate samples (10–100 μ l) were assayed for residual activity. Incorporation of label was monitored by a filter-disc method (Kleanthous *et al.*, 1985) and by gel filtration. For the filter-disc method samples (50 μ l) were applied to 2.5 cm-diameter circles of Whatman 3MM filter paper and the protein was precipitated by immersion in ice-cold 5% (w/v) trichloroacetic acid. Unbound radioactive material was removed by washing the discs with fresh ice-cold 5% 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 drying. The filter-disc radioactivities were counted and corrections made for quenching by the paper and the protein applied to it. For the gel-filtration method samples (100 μ l) were filtered through a column (30 cm $\times$ 1.5 cm) of Sephadex G-50 at 99 ml/h. Fractions (3 ml) were collected, and the location and concentration of protein were determined by the method of Bradford (1976), with BSA as standard. The amount of radiolabel incorporated was determined by counting the radioactivities of samples (500 μ l) of the fractions containing protein.

To identify the site(s) modified by D(-)-bromoacetyl-mandelate, D(-)-mandelate dehydrogenase (5 mg in 1.3 ml of 50 mM-Tris/HCl buffer, pH 8.0) was incubated with 14 C-labelled D(-)-bromoacetylmandelate (2.4 mM) and acetonitrile (0.73 M) until only 15% of the original activity remained. Labelled enzyme was removed from unbound radioactive material by gel filtration as described above, and the fractions containing protein were pooled and freeze-dried. The modified protein was then dissolved in 1 ml of 0.1 M-Tris/HCl buffer, pH 8.5, containing 6 M-guanidinium chloride and 28 mM-2-mercaptoethanol, and stirred for 2 h at 37 °C. Then 110 μ l of 1 M-NaOH was added and the mixture was stirred for 6 h at 37 °C to ensure cleavage of the protein-bound ester. The protein was separated by gel filtration as described above, and fractions containing protein were pooled and freeze-dried. Finally, the modified protein was suspended in 3 ml of 50 mM-ammonium bicarbonate buffer, pH 8.5, and trypsin (10 mg/ml in 0.1 mM-HCl) was added at a ratio of 1:50 (w/w). The digest was stirred for 4–5 h at 37 °C, after which the mixture was freeze-dried. Peptides were separated by reverse-phase h.p.l.c. as described in Fig. 5 legend.

Determination of peptide amino acid composition and sequence

For amino acid analysis, freeze-dried peptide was suspended in 6 M-HCl (200 μ l) containing 1% (w/v) phenol, and the tube was evacuated, sealed and incubated at 110 °C for 24 h. After hydrolysis the HCl was removed *in vacuo*, the amino acids were treated with phenyl isothiocyanate, and the phenylthio-carbamoyl-amino acids were then separated by reverse-phase h.p.l.c. (Waters PicoTag). Peptides were sequenced by using an Applied Biosystems 477A liquid-pulse sequencer equipped with an on-line amino acid phenylthiohydantoin analyser and by automated solid-phase Edman degradation (Laursen, 1971) in the microsequence facility built in Leeds. A full description of the solid-phase sequencing protocol has been given by Findlay *et al.* (1989).

Thin-layer electrophoresis of amino acids

D(-)-Mandelate dehydrogenase (800 μ g, 21 nmol) inactivated with 14 C-labelled D(-)-bromoacetylmandelate was acid-hydrolysed for 24 h and freeze-dried, and 4 nmol samples (5000 c.p.m.) were spotted on to 20 cm $\times$ 20 cm t.l.c. plates

(Polygram Sil G silica) along with neutral, basic and carboxymethylated amino acid standards. Plates were electrophoresed in a Shandon flat-bed electrophoresis tank with a 10% (v/v) pyridine/0.5% (v/v) acetic acid buffer, pH 6.5. Plates were then air-dried, washed once with acetone and then twice with acetone containing 1% (v/v) triethylamine, and then stained with acetone containing 0.01% fluorescamine. The amino acid spots were detected by their fluorescence under u.v. light. Plates were autoradiographed, and then the lane containing the enzyme hydrolysate was cut into 40 0.5 cm strips and the 14 C radioactivity of each strip was counted.

Synthesis of (4S)-[4- 3 H]NADH

This method relies on the fact that *L. mesenteroides* glucose-6-phosphate dehydrogenase specifically transfers a hydride ion from glucose 6-phosphate to the *si*-face of the dihydronicotinamide ring of NAD $^{+}$ or NADP $^{+}$ (Arnold *et al.*, 1976). Tritiated glucose 6-phosphate was produced by phosphorylation of D-[1- 3 H]glucose by ATP with the use of *S. cerevisiae* hexokinase. The linked assay was carried out in a mixture (500 μ l) containing 5 mM-potassium phosphate buffer, pH 8.0, 60.5 μ M-D-[1- 3 H]glucose (specific radioactivity 1.9 Ci/mmol), 500 μ M-ATP, 3.7 units of hexokinase, 60.5 μ M-NAD $^{+}$ and 55 units of glucose-6-phosphate dehydrogenase. The reduction of NAD $^{+}$ was monitored at 340 nm. When the progress curve reached a plateau, protein was removed from the mixture by ultrafiltration on a Centricon-10 microconcentrator (Amicon).

Synthesis of (4R)-[4- 3 H]NADH

S. cerevisiae alcohol dehydrogenase specifically removes the *pro-R*-hydrogen atom from NADH (Fisher *et al.*, 1953; Vennesland, 1956). [4- 3 H]NAD $^{+}$ was produced by incubating (4S)-[4- 3 H]NADH with alcohol dehydrogenase and acetaldehyde. The [4- 3 H]NAD $^{+}$ was then incubated with non-radioactive glucose 6-phosphate and glucose-6-phosphate dehydrogenase, which transfers a hydride ion to the *si*-face of the ring, thereby producing (4R)-[4- 3 H]NADH. To a filtered reaction mixture containing (4S)-[4- 3 H]NADH (prepared as above) was added 1 M-acetaldehyde (10 μ l) and alcohol dehydrogenase (10 units). The oxidation of NADH was monitored at 340 nm. When the progress curve reached a plateau, protein was removed from the mixture by using a Centricon-10 microconcentrator. The tritiated NAD $^{+}$ was then purified by h.p.l.c. on an LKB DEAE-5PW Glas-Pac column (75 mm $\times$ 8 mm) run isocratically with 10 mM-formic acid to elute NAD $^{+}$, followed by a linear 0–500 mM-NaCl gradient to elute ADP and ATP. Fractions containing tritiated NAD $^{+}$ were collected and freeze-dried. (4R)-[4- 3 H]NADH was then produced in a mixture (500 μ l) containing 10 mM-potassium phosphate buffer, pH 8.0, 8.4 μ M-[4- 3 H]-NAD $^{+}$, 50 μ M-glucose 6-phosphate and 28 units of glucose-6-phosphate dehydrogenase. The reaction was monitored at 340 nm and the protein was removed as above.

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

To a reaction mixture (1 ml) containing 10 mM-potassium phosphate buffer, pH 7.0, 100 μ M-NADH and 100 μ M-phenylglyoxylate was added either (4S)-[4- 3 H]NADH (1.2 nmol, 2.3 μ Ci) or (4R)-[4- 3 H]NADH (0.84 nmol, 1.6 μ Ci). The reaction was initiated by the addition of D(-)-mandelate dehydrogenase (3.5 μ g, 92 pmol), and once the reaction was at equilibrium the protein was removed by using a Centricon-10 microconcentrator. The NAD $^{+}$ and D(-)-mandelate were separated by h.p.l.c. Samples (500 μ l) were injected into a Brownlee Laboratories C $_8$ RP-300 reverse-phase h.p.l.c. column (220 mm $\times$ 4.6 mm). The compounds were eluted with 0.1% trifluoroacetic acid at

Fig. 2. Inactivation of D(-)-mandelate dehydrogenase by various concentrations of D(-)-bromoacetylmandelate in the absence and in the presence of D(-)-mandelate

(a) Inactivation of D(-)-mandelate dehydrogenase in the absence of D(-)-mandelate. The enzyme was incubated as described in the Experimental section in the presence of 4 mM- (●), 3 mM- (○), 2 mM- (△), 1.5 mM- (▲) and 1 mM- (□)-D(-)-bromoacetylmandelate. (b) Inactivation of D(-)-mandelate dehydrogenase in the presence of 5 mM-D(-)-mandelate. The enzyme was incubated as described in the Experimental section in the presence of 8 mM- (●), 4 mM- (○), 3 mM- (△), 2 mM- (▲) and 1 mM- (□)-D(-)-bromoacetylmandelate.

1 ml/min and the eluate was monitored at 220 nm and 260 nm. Fractions were collected and counted for tritium radioactivity.

RESULTS

Kinetics of inactivation of D(-)-mandelate dehydrogenase by D(-)-bromoacetylmandelate

In preliminary experiments D(-)-mandelate dehydrogenase was found to be inactivated by D(-)-bromoacetylmandelate, and incubation of the enzyme with 8 mM-D(-)-bromoacetylmandelate typically resulted in greater than 95% inactivation after 2 h at 27 °C. When the enzyme was incubated

under the same conditions in the absence of the inhibitor but in the presence of 0.95 M-acetonitrile (the concentration in the inactivation mixture) the enzyme remained fully active, indicating that the solvent alone did not inactivate the enzyme. Moreover, when enzyme inactivated with D(-)-bromoacetylmandelate was dialysed extensively against 50 mM-Tris/HCl buffer, pH 8.0, there was no restoration of enzymic activity, indicating that the inhibitor was covalently bound.

The inactivation of D(-)-mandelate dehydrogenase at various inhibitor concentrations in the absence and in the presence of D(-)-mandelate followed pseudo-first-order kinetics (Fig. 2). A plot of half-time of inactivation ($t_{1/2}$) against the reciprocal of the D(-)-bromoacetylmandelate concentration in the absence of D(-)-mandelate gave a straight line with a positive ordinate intercept, indicating that the inactivation of D(-)-mandelate dehydrogenase obeyed rate-saturation kinetics (Fig. 3; Meloche, 1967). D(-)-Bromoacetylmandelate therefore forms a dissociable complex with the enzyme before inactivation. The finite inactivation half-time ($t_{min.}$) was 22.7 min (21.1 and 24.3 min) and the inhibitor concentration giving the half-maximal rate of inactivation (K_i) was 2.4 mM (2.4 and 2.3 mM) (Fig. 3). The affinity of D(-)-bromoacetylmandelate 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 an apparent K_i of 4.8 mM, indicating that D(-)-bromoacetylmandelate and D(-)-mandelate compete for the same site. The ordinate intercept increased slightly in this experiment (Fig. 3), resulting in a finite inactivation half-time ($t_{min.}$) of 29.1 min, although in other experiments (results not shown) the ordinate intercepts of secondary plots in the absence and in the presence of D(-)-mandelate remained unchanged, indicating that this observed difference was probably not significant.

Fig. 3. Secondary plots of half-time of inactivation ($t_{1/2}$) as a function of the reciprocal of the D(-)-bromoacetylmandelate concentration

The half-times ($t_{1/2}$) of inactivation of D(-)-mandelate dehydrogenase at several concentrations of D(-)-bromoacetylmandelate were determined from the primary plots shown in Fig. 2. The enzyme was inactivated in the presence of inhibitor in the absence (○) and in the presence (●) of 5 mM-D(-)-mandelate.

Stoichiometry and site of modification by ^{14}C -labelled D(-)-bromoacetylmandelate

Radiolabelled D(-)-bromoacetylmandelate was synthesized in order to determine the stoichiometry and site of modification

of the enzyme. However, a different synthetic route had to be adopted so that radioactivity could be incorporated into the inhibitor (see the Experimental section). The kinetics of inhibition by the radiolabelled reagent were very similar to those with the unlabelled compound (results not shown). When D(-)-mandelate dehydrogenase was incubated with ^{14}C -labelled D(-)-bromoacetylmandelate, radioactivity (i.e. from the acetyl moiety) was incorporated into the protein. The incorporation of radioactivity into the enzyme was proportional to the loss of enzyme activity and, when extrapolated to zero activity, corresponded to the incorporation of 1.27 mol of label/mol of enzyme subunit (Fig. 4).

The labelling of D(-)-mandelate dehydrogenase was scaled up and the protein was digested with trypsin as described in the Experimental section. Peptides were purified by h.p.l.c. on a Waters $\mu\text{Bondapak C}_{18}$ reverse-phase column equilibrated with 0.1% trifluoroacetic acid in water/acetonitrile (9:1, v/v) and developed with a gradient of 0.1% trifluoroacetic acid in acetonitrile, which yielded two major radioactive peaks (Fig. 5). Protection experiments in which D(-)-mandelate dehydrogenase was inactivated with ^{14}C -labelled D(-)-bromoacetylmandelate in the presence of D(-)-mandelate showed that incorporation of radiolabel into these two sites was specifically lowered in the presence of the substrate (results not shown). However, the relative distribution of the radioactive peaks 1 and 2 was dependent upon the duration of the trypsin digestion. In one experiment the enzyme was digested for 4 h, and this resulted in peak 2 containing approximately twice the amount of radioactivity that was in peak 1 (results not shown), whereas in a second experiment the enzyme was digested for 5 h, which resulted in peak 1 containing approximately double the amount of radioactivity that was in peak 2 (as in Fig. 5).

The first radioactive peak (tryptic-digest peptide 1) was further purified by h.p.l.c. on a Waters $\mu\text{Bondapak C}_{18}$ reverse-phase column equilibrated with 1 mM-potassium phosphate buffer (pH 7.4)/acetonitrile (9:1, v/v) and developed with a gradient of acetonitrile (to 3:2, v/v) in 20 min. The peptide was finally purified to homogeneity and simultaneously desalted with the same column equilibrated with 0.1% trifluoroacetic acid in water/acetonitrile (9:1, v/v) and developed with a gradient of 0.1% trifluoroacetic acid in acetonitrile (to 1:1, v/v) in 25 min. This resulted in the elution of a single peak of radioactivity, which coincided with a single peak of absorbance at 220 nm. Tryptic-digest peptide 1 was successfully purified twice, once each from two independent batches of inactivated enzyme.

The amino acid composition of tryptic-digest peptide 1 showed that it contained glutamic acid/glutamine, glycine, valine, isoleucine, leucine, lysine and an unidentified acidic residue. Liquid-pulse sequencing (Table 1) indicated that the peptide was seven residues in length and that the glutamic acid/glutamine identified by amino acid analysis was glutamic acid. The unidentified residue at position 2 was eluted from the column before the standard *S*-carboxymethylcysteine, confirming its acidic nature, but also indicating that the single cysteine residue of D(-)-mandelate dehydrogenase (Baker & Fewson, 1989) is not the primary site of modification. In addition to the unknown residue at position 2, solid-phase sequencing could identify neither the *N*-terminal residue nor the lysine residue at position 7, as they remained bound to the support matrix via their α -amino and ϵ -amino groups respectively. The radiolabel was eluted with the unidentified residue at position 2 with solid-phase sequencing, although the radioactivity was too low to be more than indicative of this residue being labelled. More significant amounts of radioactivity were detected when the liquid-pulse sequencer was used with a larger sample, and although there was a spread of radiolabel in several fractions it did not begin to be eluted until

Fig. 4. Incorporation of ^{14}C from ^{14}C -labelled D(-)-bromoacetylmandelate into D(-)-mandelate dehydrogenase as a function of the residual enzymic activity

Incorporation of ^{14}C into D(-)-mandelate dehydrogenase was measured by using a filter-disc method (O) and gel filtration (●) as described in the Experimental section.

Fig. 5. Reverse-phase h.p.l.c. chromatograph of a tryptic digest of inactivated D(-)-mandelate dehydrogenase

Tryptic-digest peptides (2.5 nmol) prepared as described in the Experimental section were loaded on to a Waters $\mu\text{Bondapak C}_{18}$ reverse-phase h.p.l.c. column equilibrated with 0.1% trifluoroacetic acid in water/acetonitrile (9:1, v/v) and developed with a gradient of 0.1% trifluoroacetic acid in acetonitrile in 60 min (----) at a flow rate of 1 ml/min. A_{220} was monitored continuously and 1 ml fractions were collected. The location of the radiolabel was determined by scintillation counting. During large-scale injection of peptides (13.2 nmol) the two major peaks were collected manually.

the second residue had been cleaved off. This spread of radiolabel can be attributed to the poor recovery of highly polar species from the reaction chamber. In the light of both of the results it seems likely that the radiolabel had been incorporated into the residue at position 2.

The second radioactive peak (tryptic-digest peptide 2) was further chromatographed on a Waters $\mu\text{Bondapak C}_{18}$ reverse-phase h.p.l.c. column equilibrated with 0.05% ammonium acetate buffer (pH 6.0)/acetonitrile (4:1, v/v) and developed with a gradient of acetonitrile (to 1:1, v/v) in 30 min. The final purification and desalting step involved the same column equi-

Table 1. Recovery of radiolabel and sequence of tryptic-digest peptide 1 from ^{14}C -labelled D(-)-mandelate dehydrogenase

The sequence of tryptic-digest peptide 1 was determined as described in the Experimental section. Approx. 0.2 nmol of peptide (400 c.p.m.) was sequenced by using the solid-phase sequencer, and approx. 2 nmol (4000 c.p.m.) was sequenced by using the liquid-pulse sequencer. The amino acid phenylthiohydantoin derivative identified at each cycle is given, along with the amount recovered in pmol. Abbreviation: Unk, residue unknown.

Cycle	Amino acid phenylthiohydantoin derivative	Amount (pmol)	Radioactivity (c.p.m. above background)
Solid-phase sequencing			
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			
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

Fig. 6. Thin-layer electrophoresis of an acid hydrolysate of D(-)-mandelate dehydrogenase inactivated with ^{14}C -labelled D(-)-bromoacetylmandelate

The preparation of samples for thin-layer electrophoresis is described in the Experimental section. Lane 1, 10 nmol of arginine; lanes 2 and 5, 100 nmol mixture of (from right to left) histidine, *N*-carboxymethylhistidine, *N*-carboxymethylhistidine, *N,N*-dicarboxymethylhistidine; lane 3, 10 nmol of *S*-carboxymethylcysteine; lane 4, 4 nmol of D(-)-mandelate dehydrogenase hydrolysate; lane 6, 10 nmol of histidine; lane 7, 13 nmol of glycine. The hatched area represents the most radioactive portion of the plate as determined by autoradiography.

librated with 0.1% trifluoroacetic acid in water/acetonitrile (7:3, v/v) and developed with a gradient of 0.1% trifluoroacetic acid in acetonitrile (to 2:3, v/v) in 30 min. This second radioactive peak contained two peptides. One was a tetrapeptide that

contained no radiolabel and the other was a much longer peptide, and they presumably represented the shoulder and the peak respectively seen in the A_{220} elution profile. Liquid-pulse sequencing showed that none of the first 14 residues of the longer peptide contained ^{14}C . Further, this sequence did not end with lysine or arginine, as would be expected at the C-terminus of a tryptic-digest peptide. The most likely explanation is that this peptide is an *N*-terminally extended version of peptide 1 and that it was washed out of the sequencing system (because of its hydrophobic nature) before an overlap with peptide 1 was reached.

In an attempt to determine the identity of the modified residue, D(-)-mandelate dehydrogenase inactivated with ^{14}C -labelled D(-)-bromoacetylmandelate was acid-hydrolysed, a procedure that converts α -(halo ester)-modified amino acids into their carboxymethylated derivatives (Adamson *et al.*, 1984). The amino acids were electrophoresed as described in the Experimental section, and a typical plate is illustrated in Fig. 6. Although more than one type of amino acid had been modified, as indicated by the presence of radiolabel in several strips, the major peak co-migrated with the standard *N*-carboxymethylhistidine, indicating that a histidine residue at the active site of D(-)-mandelate dehydrogenase is the most likely nucleophile with which ^{14}C -labelled D(-)-bromoacetylmandelate reacts.

Stereospecificity of hydride transfer

The stereospecificity of hydride transfer was determined by measuring the ratio of label (^3H) in NAD^+ and D(-)-mandelate after the enzyme had been incubated in the presence of phenylglyoxylate and either (4*S*)-[4- ^3H]NADH or (4*R*)-[4- ^3H]NADH. When D(-)-mandelate dehydrogenase was incubated with (4*S*)-[4- ^3H]NADH and phenylglyoxylate the proportions of total radioactivity incorporated into the resulting NAD^+ and D(-)-mandelate were 95.7% and 4.3% respectively, indicating that the enzyme transferred the *pro-R*-hydrogen atom, leaving the tritium in the cofactor. The result was confirmed when the enzyme was incubated with (4*R*)-[4- ^3H]NADH and phenylglyoxylate, where the proportions of total radioactivity incorporated into the NAD^+ and D(-)-mandelate were 2.4% and 97.6% respectively. Final confirmation that the label from the (4*R*)-[4- ^3H]NADH was covalently incorporated into an unexchangeable position of D(-)-mandelate was obtained after recrystallization. The tritiated D(-)-mandelic acid (44 nmol, 0.7 μCi) purified by h.p.l.c. was diluted with non-radioactive D(-)-mandelic acid (678 mg, 4.5 mmol) and recrystallized from toluene. The crystals were washed thoroughly with ice-cold pentane and dried under high vacuum. Recrystallization was repeated twice and the specific radioactivities of D(-)-mandelic acid were 131 $\mu\text{Ci/mol}$ and 130 $\mu\text{Ci/mol}$, i.e. it had recrystallized with a constant specific radioactivity.

DISCUSSION

For a compound to be considered as an affinity label or active-site-directed inhibitor it must satisfy the following criteria: It should inactivate the enzyme completely and irreversibly, the inactivation should show rate saturation, the inactivation should be protected by the substrate or by reversible inhibitors of the enzyme, and the covalent attachment of the inhibitor should be stoichiometric. D(-)-Bromoacetylmandelate 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 active-site-directed nature of the inactivation by D(-)-bromoacetylmandelate was demonstrated clearly by the pro-

<i>Ps. putida</i> L(+) -mandelate dehydrogenase	T ³⁸⁰ APVDHL-IGKGTHA ³⁹³ -OH
<i>R. graminis</i> D(-) -mandelate dehydrogenase	V-XLEIGK
<i>Ps. putida</i> mandelate racemase	I ³⁴⁸ WREKEIGKYLY ³⁵⁷ -OH

Fig. 7. Sequence alignment of tryptic-digest peptide 1 with the extreme C-termini of *Ps. putida* mandelate racemase and L(+) -mandelate dehydrogenase

Information is taken from the present paper, Ransom *et al.* (1988) and Tsou *et al.* (1990). Gaps have been introduced into two of the sequences in order to increase the degree of apparent sequence similarity and to indicate the possible alignment of the histidine residue of *Ps. putida* L(+) -mandelate dehydrogenase with the putative histidine residue at the active site of *R. graminis* D(-) -mandelate dehydrogenase.

tection afforded by D(-)-mandelate at concentrations of inhibitor approximating to the K_m for the substrate (Fig. 2); the apparent K_m for D(-)-mandelate at pH 8.0 is 0.37 mM (Baker, 1990) and the K_i for D(-)-bromoacetylmandelate at the same pH is 2.4 mM. Although it might have been expected that 5 mM-D(-)-mandelate would have afforded more substantial protection than was observed, it has to be remembered that the protection experiments were carried out in the absence of added NAD⁺ whereas the determination of the apparent K_m for D(-)-mandelate was done in the presence of NAD⁺. The alkylating agents iodoacetate and iodoacetamide do not inhibit the enzyme (Baker & Fewson, 1989), yet they possess a similar electrophilic group to D(-)-bromoacetylmandelate. This observation underlines the importance of structural features necessary for specific association and orientation of the bromoacetyl moiety at the mandelate-binding site/active site in order to allow alkylation to occur; these features are presumably missing from the simple group-specific alkylating agents.

The separation of tryptic peptides of D(-)-mandelate dehydrogenase inactivated with ¹⁴C-labelled D(-)-bromoacetylmandelate resulted in two main peaks of radioactivity. The longer digestion produced a shift in radiolabel from peak 2 to peak 1, and presumably digestion of the inactivated enzyme for even greater lengths of time would have resulted in the complete loss of radiolabel from peak 2 as the partial digest product became fully cleaved. Therefore both peptides are probably related to one another and almost certainly represent partial-cleavage products from the original tryptic digestion. This notion is supported by the stoichiometry of inactivation, which showed that essentially only one site was labelled by D(-)-bromoacetylmandelate (Fig. 4). Unfortunately the complete sequence of the longer tryptic-digest peptide could not be determined.

The inability to detect S-carboxymethylcysteine by peptide sequencing or electrophoresis of the modified amino acids, coupled with the marked insensitivity of the enzyme to a range of thiol-blocking reagents (Baker & Fewson, 1989) seems to rule out a direct role for the single cysteine side chain in the catalytic mechanism of D(-)-mandelate dehydrogenase. Of the other likely residues that could participate in donating a proton to the carbonyl oxygen atom of phenylglyoxylate, histidine seems the most probable candidate, as the main peak of radiolabel, from a hydrolysate of inactivated enzyme, co-migrated with the standard N^ε-carboxymethylhistidine (Fig. 6). Furthermore, other NAD⁺-dependent 2-hydroxy acid dehydrogenases such as lactate dehydrogenase and malate dehydrogenase are known to have an active-site histidine residue that acts as an acid/base catalyst and, as part of a histidine-aspartic acid couple, participates in proton donation (Birktoft & Banaszak, 1983; Roderick & Banaszak, 1986; Clarke *et al.*, 1988). Further evidence to support a similar reaction mechanism for D(-)-mandelate dehydrogenase, lactate dehydrogenase and malate dehydrogenase is the common transfer of the *pro-R*-hydrogen atom of NADH to the 2-oxo acid substrate. Malate dehydrogenases from a diverse range of organisms, including archaeobacteria, eubacteria, fungi, plants,

arthropods, birds and mammals, all transfer the *pro-R*-hydrogen atom, as do lactate dehydrogenases from an equally diverse range of organisms (You, 1982; Görisch *et al.*, 1985). Consequently these similarities afford a plausible explanation as to the origin and evolution of D(-)-mandelate dehydrogenase. Recruitment of an NAD⁺-dependent 2-hydroxy acid dehydrogenase by duplication and/or mutation of an existing gene may have provided an enzyme with an established NAD⁺-dependence and a reaction mechanism capable of oxidizing a 2-hydroxy acid, and therefore relatively few mutations at the substrate-binding site may have been required to endow the resulting enzyme with the ability to use D(-)-mandelate as substrate.

Recruitment of the enzyme to the mandelate pathway of *R. graminis*, even though it functions against the equilibrium of the reaction (Baker & Fewson, 1989), presumably represented a strong selective advantage in enabling the yeast to grow on an additional carbon source. Similarly, 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 that normally catalyses the oxidation of lactate. Mutants that lack this enzyme can still grow on lactate since they retain a cytoplasmic NAD⁺-dependent lactate dehydrogenase, which 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).

During the course of the present work we noticed that the sequence of the D(-)-mandelate dehydrogenase active-site peptide was similar to sequences in the extreme C-termini of both the mandelate racemase and the membrane-bound L(+) -mandelate dehydrogenase of *Ps. putida* (Fig. 7). However, we doubt whether this is indicative of a common functional role because none of the corresponding residues in mandelate racemase have been implicated in substrate binding or catalysis (Neidhart, 1989). We have not detected any other significant similarities between the amino acid sequence of L(+) -mandelate dehydrogenase from *Ps. putida*, which is known in its entirety (Tsou *et al.*, 1990), and those of any of the peptides from the D(-)-mandelate dehydrogenase of *R. graminis* that we have sequenced.

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