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Mandelic Acid Racemase from *Pseudomonas putida*. Purification and Properties of the Enzyme*

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ABSTRACT: Mandelic acid racemase (EC 5.1.2.2.) is an inducible enzyme of limited biological distribution that enables *Pseudomonas putida* A.3.12 to use both enantiomers of mandelic acid as source of carbon and energy. Extracts prepared from induced cells yield preparations of enzyme homogeneous by disc gel electrophoresis after 550-fold purification. Antisera prepared against the purified protein give a single band of precipitation in gel diffusion tests with crude extracts of induced cells. The enzyme has a molecular weight of about 200,000 and does not contain dissimilar subunits. The purified enzyme is relatively resistant to a wide range of inhibitors and exhibits no unusual regulatory behavior. Its spectral properties suggest that it does not function with a flavin or pyridine nucleotide cofactor. A number of compounds of structure analogous to mandelic acid were tested as substrates for the racemase. α-Hydroxy acids with a phenyl or monosubstituted phenyl substituent on the α-carbon and imidazolelactic acid were the most reactive substrates. Mandelic acids which were disubstituted on the phenyl ring were racemized relatively slowly.

Reversible inhibition by a number of unreactive analogs of mandelic acid was observed. The rates of racemization of several mandelic acid derivatives monosubstituted in the para position were measured. The relative rates indicated that stabilization of negative charge on the α-carbon facilitated the racemization.

A number of microorganisms able to use mandelic acid as sole carbon and energy source are known (Table I). Of these, the only bacterium known able to oxidize both isomers of mandelate is *Pseudomonas putida* A.3.12 (ATCC 12633). Gunsalus and coworkers (1953b) showed that this unique ability reflects the possession by this strain of a racemase capable of interconverting the D-(−) and L-(+) enantiomers of mandelic acid. The other bacteria possess a single stereospecific mandelic dehydrogenase and thus, with *P. putida* A.3.12 as the only known exception, can grow only at the expense of either the D or L isomer of mandelic acid.

P. putida A.3.12 metabolizes D-(−)-mandelic acid and D-(−)-4-hydroxymandelic acid *via* a pathway comprising a sequence of at least 11 specifically inducible enzymes (Figure 1). The first five of these enzymes, termed the *mandelate group*, act to convert D-(−)-mandelate into benzoate (Gunsalus *et al.*, 1953b) and are specifically inducible by D-(−)-mandelate, L-(+)-mandelate, or benzoylformate (Hegeman, 1966a). The benzoate or 4-hydroxybenzoate that is formed is metabolized *via* the *β-ketoadipate pathway* (Ornston and Stanier, 1966). Since the mandelate group is coordinately inducible under gratuitous conditions (Hegeman, 1966b), is constitutively synthesized by a regulatory mutant (Hegeman, 1966c), and at least five of the six genes that specify it may be cotransduced with high frequency (Chakrabarty and Gunsalus, 1969), the group probably constitutes an operon.

We wish to report the purification and characterization of the first enzyme of the mandelate group, mandelic acid racemase (EC 5.1.2.2). At the time of its first description and partial purification (Gunsalus *et al.*, 1953a) it was established that mandelic acid racemase was a soluble enzyme that appeared to function without a known cofactor. A subsequent examination of mandelate racemase by Weil-Malherbe (1966) revealed a number of interesting properties. Among these is an increase in the affinity for the substrate caused by certain divalent metal ions. However, this work was carried out with only partially purified material and, for the

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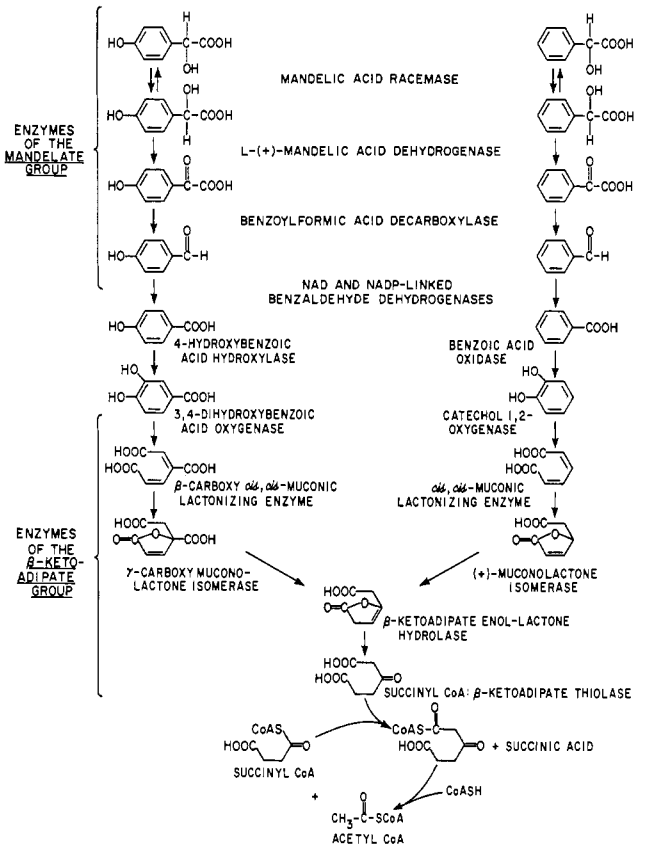


FIGURE 1: Pathway for oxidation of DL-mandelic acid and DL-4-hydroxymandelic acid by *P. putida* A.3.12. The same group (the mandelate group) of five coordinate enzymes acts to convert the primary substrates into the corresponding benzoic acids.

most part, also relied on a complex multienzyme coupled assay.

The observation that mutants blocked in mandelic acid racemase or mandelic acid dehydrogenase (Hegeman, 1966b) are also either impaired in growth or fail to grow at the expense of DL-4-hydroxymandelic acid (G. D. Hegeman, 1970, unpublished data) confirmed the findings of Gunter (1953) and Stanier *et al.* (1953) which indicated that the mandelate group functions in the metabolism of these compounds by *P. putida*. These findings indicate a broader specificity than previously suspected (Gunsalus *et al.*, 1953a). Only brief surveys of the kinetic properties and substrate specificity had been reported previously (Gunsalus *et al.*, 1953a; Weil-Malherbe, 1966); therefore we considered it desirable to confirm and extend these observations using the highly purified protein. It was hoped that these specificity and kinetic studies would be helpful in elucidating the mechanism of action of the enzyme.

Experimental Procedures

Biological Methods and Materials. Cells of *P. putida* A.3.12 (PRS 1, ATCC 12633) were grown at 30° with aeration in mineral medium (Hegeman, 1966a) containing 0.2–0.4% ammonium DL-mandelate as sole carbon and energy source. Stock cultures were maintained on solid medium containing 1% yeast extract and 1.5% agar (both Difco Laboratories

TABLE I: Microorganisms Capable of Growth at the Expense of Mandelic Acid.

Strain of (Organism)	Isomers Used	References
Pseudomonas putida	L(+), D(−), D and L (1 strain)	Gunsalus *et al.* (1953a)
P. aeruginosa	L(+)	Stanier *et al.* (1966)
P. fluorescens	D(−), L(+)	
Azotobacter beijerinckii[a]	D(−), L(+)	
Bacillus sphaericus[a]	L(+)	
Acinetobacter NCIB 8250	L(+)	Kennedy and Fewson (1968)
Aspergillus niger[b]	D and L	Jamaluddin *et al.* (1970)
Yeast[a] (unidentified)	L(+)	

[a] Isolated by standard enrichment techniques from soil and water in the authors' laboratory. [b] *Aspergillus* does not possess a racemase but has two oxidases, each of different stereospecificity (Jamaluddin *et al.*, 1970).

products). A strain of *P. putida* (ATCC 17426) which closely resembles *P. putida* A.3.12 was grown in the same way and used as a source of D-(−)-mandelic acid dehydrogenase.

Racemase-free L-(+)-mandelic acid dehydrogenase [no IUB EC number, systematic name: L-(+)-mandelate: (acceptor) oxidoreductase] was prepared by twice sedimenting (100,000*g* for 60 min) and resuspending the *particle fraction* (Hegeman, 1966a) from cells of the *rac*⁻ (PRS 2) mutant derived from strain A.3.12 (Hegeman, 1966b) grown on benzoylformic acid. The enzyme was measured and stored as previously described (Hegeman, 1966a).

Neurospora crassa NADase (3.2.2.5) was the product of the Worthington Biochemical Corp.

Antisera were prepared using antigen made by emulsification of *purified racemase* (250 μg, specific activity 190 or better) in 2 ml of Freund's complete adjuvent (Difco Laboratories, Inc.) containing 250 μg of methylated bovine serum albumin (Mandell and Hershey, 1960). New Zealand White rabbits received weekly intradermal injections for 4 weeks. In the fifth week 250 μg of enzyme was injected intravenously. This injection schedule was repeated until high titers of precipitating antibody were apparent in sera obtained by sample bleeding from the ear. Serum was collected from blood obtained by cardiac puncture and stored frozen at −20°.

Chemical Methods and Materials. DL-Mandelic acid and phenylacetic acid were purchased from Matheson, Coleman and Bell, Inc. Phenylacetic acid was recrystallized twice from ethanol–water before use. Phenyloxyacetic acid, DL-4-chloromandelic acid, and DL-α-methoxyphenylacetic acid were the products of Eastman Organic Chemicals, Inc. DL-4-Bromomandelic acid, thiophenoxyacetic (phenylmercaptoacetic) acid, benzoic acid, and D-(−)- and L-(+)-mandelic acids were obtained from the Aldrich Chemical Co. DL- and L-β-phenyllactic acids were the products of Pfaltz and Bauer Co. DL-4-Hydroxymandelic acid and DL-4-methoxymandelic acid were obtained

from K and K Laboratories. DL-3,4-Dihydroxymandelic acid and DL-4-hydroxy-3-methoxymandelic (vanillylmandelic) acids were purchased from Calbiochem Corp. DL-3-Indolelactic and L-β-imidazolelactic acids were obtained from the Sigma Chemical Co. DL-Atrolactic acid (hemihydrate) was purchased from Beacon Chemical Industries, Inc. D-(−)- and L-(+)-lactic acids (as the lithium salts) were the products of Miles Laboratories, Inc. Diphenylacetic acid was obtained from Mann Laboratories, Inc.

The sodium salts of the pure D isomers of the substituted mandelic acids were prepared by specific enzymatic oxidation of the L isomers to the corresponding α-keto acids in 0.1 M Na_2HPO_4–KH_2PO_4 buffer (pH 6.8) at 30° by use of L-(+)-mandelic acid dehydrogenase [*particle fraction* (Hegeman, 1966a)] prepared from benzoylformate-grown cells of the *rac*$^-$ mutant. When the reaction was completed as judged by cessation of measurable oxygen uptake determined in a sample of the reaction mixture in a Warburg respirometer (Gilson Medical Electronics, Inc.), the particle fraction was removed by centrifugation (105,000*g* for 90 min). The D isomers were separated from the supernatant fluid following acidification with HCl (pH $\leq$ 1.0) by sequential extraction with three portions of ether equal to the volume of the acidified supernatant fluid. The combined ether extracts were reduced in volume in a rotary evaporator, or with a stream of dry N_2, and dried over anhydrous $MgSO_4$. The substituted D-mandelic acids were separated from the corresponding α-keto acids and other contaminants by preparative thin-layer chromatography using wedge plates (1000–250 μ) of silica gel (Adsorbosil-1, Applied Science Laboratories, Inc., State College, Pa.) and detected either directly by quenching of substrate fluorescence or following spray application of 0.001% Rhodamine B in acetone and examination with ultraviolet light. The adsorbent regions containing the substituted mandelic acids were collected by vacuum impingement on fritted glass funnels and the acids eluted from the silica gel with anhydrous methanol. After removal of the methanol under a stream of dry nitrogen, the purity of the acids was verified by analytical chromatography using thin-layer chromatography (125-μ thick) plates of the same type and solvent of the same composition (methanol–acetic acid–diethyl ether–benzene, 1:18:60:120, v/v) as that used in the preparative procedure. The purity of the D-substituted mandelic acids was also verified by examination of the ultraviolet spectra of aqueous solutions of their sodium salts and by gas–liquid chromatography (Kenyon and Hegeman, 1970).

Analytical Methods. Protein was determined by either the Lowry modification of the Folin method (Lowry *et al.*, 1951) or an unpublished modification by A. B. Pardee of the biuret method (Weichselbaum, 1946) using silica gel dried bovine serum albumin, fraction V (Armour Laboratories, Inc.), as reference.

The standard method of mandelic acid racemase measurement employed was that of Hegeman (1966a) modified to include 0.1 ml of 0.1 M KCN in the 3-ml reaction. This modification reduces the tendency to give spuriously low measurements with small amounts of enzyme and a nonlinear reaction time course due to electron transport system-mediated reoxidation of reduced 2,6-dichlorophenolindophenol. The assay is linear with respect to enzyme up to a rate of at least 0.25 absorbance unit/min.

Acrylamide gel disc electrophoresis was performed at pH 9.3 according to Davis (1964). Gels were stained with 1% aniline blue-black or coomassie blue in 7% acetic acid. The gels were destained by electrophoresis in 7% acetic acid. Quantitative examination of the stained gels was carried out in a Gilford Model 2410 linear transport unit fitted to a Model 2000 absorbance recorder.

Protein was reduced and carboxymethylated in some cases prior to electrophoresis according to the method of Sela *et al.* (1959). Starch gel electrophoresis was performed according to Smithies (1955, 1959) in both the prescribed buffers and in imidazolium chloride (pH 6.9), tris(hydroxymethyl)-aminomethane (Tris)–DL-histidine and sodium malonate (both pH 6.0), Tris-citrate (pH 5.0), and ammonium acetate (pH 3.5). Sliced gels were stained with 1% aniline blue-black in 5% acetic acid.

Estimation of the molecular weight of the enzyme was performed by the gel filtration method of Andrews (1964) using commercial preparations of ferritin, rabbit muscle lactic dehydrogenase (1.1.1.27), *Escherichia coli* alkaline phosphatase (3.1.3.1), horse hemoglobin, egg white lysozyme (3.2.1.17), and horse heart cytochrome *c* as reference proteins.

Immunodiffusion was by the Ouchterlony (1948) method in 1% Ionagar No. 2 (Consolidated Laboratories, Inc.) in 0.01 M phosphate-buffered saline (pH 7.2).

For measurement of the rate of enzyme-catalyzed racemization of compounds that were not substrates for L-(+)-mandelic acid dehydrogenase two alternate coupled assays other than the standard assay were employed. The first of these was identical with the standard assay except that the *particle fraction* (Hegeman, 1966a) prepared from mandelate-grown *P. putida* (ATCC 17426) replaced that derived from *P. putida* A.3.12. This strain possesses a D specific mandelic dehydrogenase and no mandelic racemase.

The second alternate coupled assay employed yeast L-lactate dehydrogenase (cytochrome b_2, EC 1.1.2.3, Worthington Biochemical Corp.) in place of mandelic acid dehydrogenase and contained all the components of the assay mixture described by Appleby and Morton (1959) in place of those contained in the standard assay. This variant was used when lactic acid and its analogs were employed as substrates. In cases where a coupled assay procedure was undesirable, measurements were made in a polarimeter (Bendix ETL-NDL, type 143A). In this case the KCN, 2,6-dichlorophenolindophenol, and mandelic dehydrogenase were omitted from the reaction mixture that was otherwise identical with that used in the standard assay.

Ultraviolet and visible spectra were measured with a Cary Model 14 recording spectrophotometer. Nuclear magnetic resonance (nmr) spectra were determined with a Varian Model T-60 spectrometer.

Purification of Mandelic Acid Racemase (*Table II*). STEP 1, CULTIVATION OF INDUCED CELLS. Inocula were prepared by growing 500-ml cultures with shaking in 2.7-l. Fernbach flasks, and two such cultures were used to inoculate 11 l. of culture medium in each of 3 Model F-14 New Brunswick fermentors. The generation time is 60 min. Growth was allowed to occur with strong aeration and rapid agitation until the culture attained a turbidity of 250 units (Klett-Summerson colorimeter fitted with a No. 66 filter). At that time roughly 0.75 of the culture was removed from the fermentor vessels and harvested in a Sharples continuous-flow

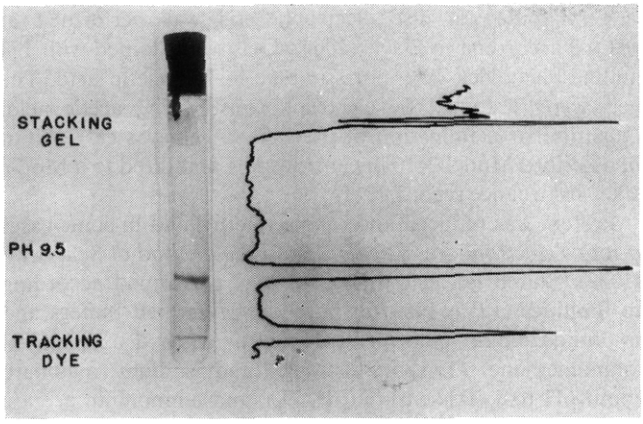


FIGURE 2: Acrylamide disc gel electrophoresis of purified mandelic racemase. A densitometric tracing appears to the right of the gel.

centrifuge. The fermentors were refilled by addition of 8 l. of new prewarmed culture medium that was prepared without aseptic precautions, and growth was allowed to resume. This process was usually repeated twice and yielded a total of 250–300 g of wet cells. The cell paste was stored frozen (−20°) prior to extraction; under these conditions loss of enzyme activity was undetectable during periods of up to 1 year.

Alternate methods of cultivation have been employed, including continuous culture in slightly modified New Brunswick fermentors. Provided that cultures are harvested before the inducer–carbon source has been exhausted, the specific cellular enzyme content is not greatly dependent upon conditions.

STEP 2, EXTRACTION. Thawed cell paste is suspended in four times its weight of 0.1 M Na_2HPO_4–KH_2PO_4 buffer (pH 6.8) by vigorous agitation. The suspension is then treated in 50-ml batches in a Raytheon (Model DF 101) 10 kHz sonic oscillator for 10 min at maximum power. Other means of extraction are also satisfactory. This and all subsequent steps were performed at 0–4°. Centrifugation (10 min, 5000*g*) sediments unbroken cells and large cellular debris, which is discarded.

STEP 3, REMOVAL OF THE PARTICLE FRACTION. The decanted supernatant fluid is then centrifuged at 105,000*g* for 90 min. The particle fraction (mostly comminuted fragments of the cell wall and membrane and the ribosomes) which sediments is discarded.[1]

STEP 4, REMOVAL OF NUCLEIC ACIDS. To the decanted supernatant fluid is slowly added 0.05 volume of 1.0 M $MnCl_2$ with stirring. The precipitate that forms over an 8-hr period is removed by repeated centrifugation (10,000*g* for 20 min) and discarded.

STEP 5, 37.5–50% $(NH_4)_2SO_4$ FRACTION. Sufficient finely ground solid $(NH_4)_2SO_4$ to attain 37.5% saturation (0°) is added slowly to the supernatant fluid with stirring. One hour after the addition is complete the precipitate that forms is removed by centrifugation (10,000*g*, 20 min) and discarded. An additional quantity of ammonium sulfate sufficient to provide 50% saturation is added as before. The precipitate that forms is collected by centrifugation as before and dissolved in a minimal amount of 0.05 M Tris-HCl (pH 8.0) containing 0.01 M $MgCl_2$ and 0.1 M NaCl (elution buffer).

TABLE II: Purification of Mandelic Acid Racemase.

Step	Fraction	Specific Activity (μmoles/min per mg)	Yield (%)	Purification (-fold overall)
1	Induced cells			
2	Crude extract	0.35	100	
3	Particle-free supernatant	0.42	96	1.2
4	$MnCl_2$ supernatant	0.82	88	2.3
5	37.5–50% $(NH_4)_2SO_4$ precipitate	2.35	83	7.0
6	Pooled fractions after gel filtration (Sephadex G-200)	50	40	143
7	Pooled fractions after DEAE-Sephadex chromatography	195	15	557

STEP 6, GEL FILTRATION.[2] The preparation is dialyzed against at least 100 volumes of elution buffer overnight and applied to a column of Sephadex (Pharmacia Fine Chemicals, Inc.) G-200 prepared by swelling and packing the gel in elution buffer. The gel bed should have a length 20 times its diameter and the sample applied should have a volume no greater than 0.1 times the volume of the gel bed. The column is eluted with the buffer used in column preparation. Central fractions containing 80% of the activity which emerges are pooled.

STEP 7, DEAE–SEPHADEX CHROMATOGRAPHY. A column of DEAE–Sephadex A-50 is prepared by swelling, packing, and equilibrating the gel with the elution buffer. The pooled Sephadex G-200 eluate is applied to the column at a ratio of not more than 1 g of protein to 20 g of dry gel. After the column is washed with several volumes of elution buffer, a linear concentration gradient of from 0.1 to 0.3 M NaCl in elution buffer is applied to elute the enzyme, which emerges in fractions containing about 0.16 M NaCl. Fractions of specific activity at least 190 are pooled. All attempts to crystallize the enzyme made to date have failed.

Properties of the Enzyme. Disc gel electrophoresis (Figure 2) and starch gel electrophoresis at several pH values revealed only one protein component in preparations of specific activity of 190 or higher. The specific activity of all fractions taken from the central portion of the peak of activity that is eluted from DEAE–Sephadex is constant within limitations of measurement. Antisera prepared against preparations of enzyme of specific activity 190 or higher give only a single band in immunodiffusion analysis with crude extracts (step 2)

[1] The particle fraction may also be saved and used as a source of L-(+)-mandelic dehydrogenase [L-(+)-mandelate: (acceptor) oxidoreductase] for assay of the racemase (Hegeman, 1966a).

[2] A heat treatment (60° for 10 min) may be used at this point and affords a threefold purification. The occurrence of occasional instability and persistent contamination following heat treatment during purifications led to its abandonment.

TABLE III: Effect of the Nature of the Substituent in the α-Position upon the Rate of Mandelic Racemase Action with Various α-Hydroxy Acids.

Substrate	Relative Rate (%)
2-Phenylglycolic acid[a] (mandelic acid)	(100)
3-Imidazolelactic acid[a,b]	5.4
3-Indolelactic acid[a,b]	≦0.01[c]
3-Phenyllactic acid[a,b]	≦0.01[c]
Lactic acid[b]	≦0.01[c]
3,4-Dihydroxyphenylglycolic acid[a]	0.03
4-Hydroxy-3-methoxyphenylglycolic acid[a] (vanillylmandelic acid)	≦0.02[c]

[a] Assays were performed using the standard assay procedure by combining with either D-(−)- or L-(+)-mandelic dehydrogenase from *P. putida*. Several substrate concentrations were used to ensure that substrate was not rate limiting. [b] Measurement performed by using the yeast lactic dehydrogenase coupled assay. [c] Limit of sensitivity of the assay procedure.

of mandelate-grown cells. The band of precipitate from the crude extract fuses in an arc of identity with the precipitate formed with purified enzyme and is not observed in crude extracts prepared from uninduced cells (Figure 3).

The enzyme migrates to the anode during starch gel and acrylamide disc gel electrophoresis at pH values from 6 to 9.5 in various buffers. The isoelectric point is therefore below 6.

The molecular weight estimated by gel filtration according to the method of Andrews (1964) is 200,000. Acrylamide disc gel electrophoresis following reduction and alkylation according to the method of Sela *et al.* (1959) revealed a single sharp band of protein of higher anodal migration rate than that exhibited by an untreated enzyme preparation.

Purified mandelic racemase exhibits no unusual light absorption properties, having a single ultraviolet absorption maximum at 278 nm. Solutions of purified enzyme (10 mg of protein/ml) exhibit no visible absorption and no fluorescence detectable to the naked eye. No significant differences in spectral properties were detected in the near ultraviolet or visible regions when solutions of the enzyme (10 mg/ml) were compared in the presence of substrate (10^{-3} M) and in its absence using double compartment cells (1 cm) and a 0.1 absorbance (full-scale) slidewire.

Purified enzyme preparations are insensitive to prolonged incubation in the presence of *N. crassa* NADase, an enzyme capable of hydrolyzing both NAD and NADP. Brief charcoal treatment does not diminish activity. Pyridine nucleotides or boiled crude extract (step 2) do not restore activity to preparations inactivated by prolonged charcoal or other treatments.

The purified enzyme exhibits a relatively broad pH optimum (pH 7.5–8.0) in Tris-HCl and phosphate buffers. The phosphate anion is a weak inhibitor of enzyme activity.

Divalent metal ions increase the affinity of the enzyme for

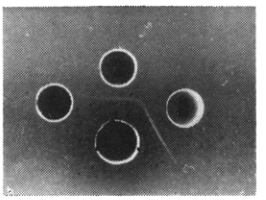


FIGURE 3: Ouchterlony diffusion pattern using antiserum (A.S.) prepared against purified mandelic racemase. Well number 1 contains crude extract prepared from uninduced cells, well number 2 contains purified mandelic racemase, and well number 3 a crude extract (step 2) of induced cells.

substrate and act as activators both in certain buffers and under conditions of substrate limitation (Weil-Malherbe, 1966). It is unlikely that divalent metals are absolutely required for activity, however, since prolonged dialysis of purified enzyme against buffers containing EDTA or pyrophosphate fails to diminish activity measured under conditions of substrate saturation in reactions from which Mg^{2+} is omitted.

N-Ethylmaleimide, *p*-chloromercuribenzoate, and iodoacetamide are weak noncompetitive inhibitors; concentrations of at least 1 mM are required to give measurable inhibition of the purified enzyme by these agents. Aromatic and nonaromatic compounds that occur as intermediates in the oxidation of mandelic acid by *P. putida* are weak inhibitors, the most potent showing measurable inhibition only at rather high (10 mM) concentrations.

Specificity of the Racemase. Various analogs of mandelic acid were investigated as possible substrates of the enzyme and the results are shown in Tables III and IV. The analogs tested were of two general chemical types, substituted phenylglycolic acids or substituted lactic acids (and lactic acid itself).

Several analogs of mandelic acid which were inactive as

TABLE IV: The Activity of Mandelic Acid Racemase upon Mandelic Acid Analogs Substituted in the 4 Position.

Analog (D Isomer) of a Mandelate Tested[a]	V_{max}[b] (μmoles/min)	Relative Rate (Unsubstituted Mandelate ≡ 1)	K_m[b] (M × 10^5)
4-Bromo	124	3.76	25.6
4-Chloro	107.5	3.26	10
4-Hydrogen (normal mandelic acid)	33	1	9.3
4-Hydroxy	14.8	0.45	29
4-Methoxy	5.5	0.17	33

[a] The standard spectrophotometric assay was employed since all the analogs tested were also substrates for L-(+)-mandelate dehydrogenase. [b] Kinetic parameters were determined according to the method of Eadie (1952) and are good to about ±20%.

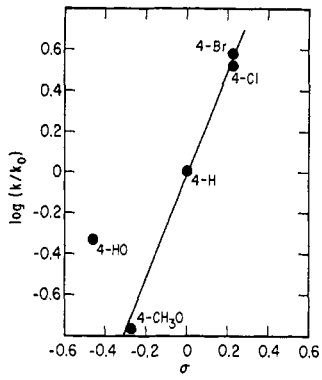


FIGURE 4: Hammett plot for some 4-substituted mandelic acids with mandelic acid racemase.

substrates of the enzyme were tested for inhibitory properties (Table V). A number of these analogs exhibited reversible inhibition if preincubated with the enzyme. In all cases the inhibition was prevented when the analogs (10^{-3} M) were added to the standard assay mixture at the same time as the substrate, D-(−)-mandelic acid (10^{-2} M).

TABLE V: Inhibition[a] of Mandelic Acid Racemase by Some Mandelic Acid Analogs.

Analog Tested	Inhibition (%)
Phenyloxyacetic acid	83
Phenylmercaptoacetic acid	83
Phenylacetic acid	74
DL-β-Phenyllactic acid	65
DL-Atrolactic acid	46
Diphenylacetic acid	19
Benzoic acid	7
DL-α-Methoxyphenylacetic acid	0

[a] The analogs (supplied as sodium salts) were each incubated with the racemase and the components of the standard coupled assay mixture at a concentration of 10^{-3} M for 30 min at 25°. The reaction was started by addition of D-(−)-mandelic acid. In no case was inhibition observed if the analogs were added after the reaction with mandelic acid had begun.

Discussion

Mandelic acid racemase may be purified 550-fold in six steps from a crude extract of *P. putida* grown at the expense of mandelic acid. The purified enzyme is a homogeneous protein preparation by acrylamide disc and starch gel electrophoresis and by immunochemical criteria. The enzyme has a molecular weight of about 200,000. Using this value and the specific activity of the most highly purified enzyme (190 units/mg), a turnover number of 1.72×10^3 sec^{-1} may be calculated. Dissimilar subunits cannot be demonstrated.

The properties of purified mandelic racemase do not differ substantially from those previously determined with crude or partially purified preparations (Gunsalus *et al.*, 1953a,b; Weil-Malherbe, 1966). The absence of strong inhibition by pathway intermediates indicates that mandelic racemase, first enzyme of the mandelate group, has no regulatory role.

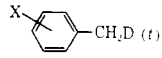
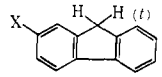
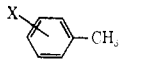
The enzyme was not resolved with respect to a cofactor during purification. This and other evidence indicates that mandelic racemase functions without a cofactor of the flavin or pyridine nucleotide type.

The specificity of the enzyme appears to be rather narrow which is in agreement with the observations made by Weil-Malherbe (1966), who tested a more limited range of substrates with a partially purified preparation of the enzyme using a complex coupled assay procedure. Of those potential substrates investigated (see Tables III and IV) only α-hydroxy acids with either a phenyl group or a monosubstituted phenyl group reacted well with the enzyme. Replacement of two hydrogens on the phenyl group in mandelic acid by substituents gave analogs (3,4-dihydroxyphenylglycolic acid and 4-hydroxy-3-methoxyphenylglycolic acid) which were racemized very slowly. We presume that the detrimental effects of these substituents are mostly steric in nature. The enzyme gave no detectable reaction with a closely related naturally occurring compound, lactic acid. Increasing the distance between the α-hydroxy acid group and the phenyl group by interposition of a methylene group (3-phenyllactic acid) also severely diminished the efficiency of the racemization, although, surprisingly, 3-imidazolelactic acid was 5% as reactive as mandelic acid.

Reversible inhibition was observed to occur when carboxylic acids having an aromatic substituent were incubated with the enzyme prior to addition of the mandelic acid. No simple structural pattern which would permit one to predict which analogs might be the best inhibitors of the racemase emerges, although bulky substituents near the carboxylate moiety appear to be unfavorable. It is interesting to note that the two most effective inhibitors of racemase activity, phenyloxyacetic acid and phenylmercaptoacetic acid, were also the only 2 of 22 nonmetabolizable analogs of mandelic acid able to act as inducers of the enzymes of the mandelate group in *P. putida* (Hegeman, 1966a). This implies that analogous structural features determine the ability to combine with the repressor governing synthesis of these enzymes and ability to bind tightly to the first enzyme of the pathway.

The relative rates of racemase action upon mandelic acid analogs substituted with either electron-withdrawing or electron-donating substituents in the 4 position of the phenyl group were investigated and the results are shown in Table IV. The tendency for the electron-withdrawing bromo and chloro substituents to enhance the V_{max} and for the electron-donating hydroxy and methoxy groups to decrease the V_{max} is apparent. Application of the Hammett ρ–σ relationship (Jaffé, 1953) to this limited data permits a kinetic ρ value of approximately +2 to be calculated for the racemase reaction. The Hammett plot of log (k/k_0) *vs.* σ is shown in Figure 4. In this calculation, however, the 4-hydroxymandelic acid is not in line with the other substituents and reacts approximately five times faster than expected. The reason for this discrepancy is not clear, although perhaps a specific hydrogen bond (or bonds) or other interaction involving a side-chain moiety of the racemase and the 4-hydroxy group diminishes the ability

TABLE VI: Representative Kinetic Hammett ρ Values.

Reactant	Reaction Studied	ρ Value	Reference
	Base-catalyzed hydrogen–deuterium (tritium) exchange	+4.0	Streitwieser and Koch, 1964
	Base-catalyzed hydrogen–tritium exchange	+3.2[a]	Streitwieser and Pudjaatmaka, 1965
	Radical bromination (hydrogen abstraction)	−1.07 to −1.78[b]	Pearson and Martin, 1963

[a] Correlated with σ_m values. [b] Correlated with σ^+ values; several brominating agents and different reaction temperatures were used.

of this substituent to behave as a donor of electron density. In any case, the results are clearly consistent with the idea that substituents which stabilize negative charge on the α-carbon in the transition state of the reaction enhance the rate of racemization. This conclusion received support from the occurrence of a large deuterium isotope effect, the existence of which indicates that the formation of an intermediate produced by cleavage of the C–H (or D) bond on the α-carbon is the rate-controlling step in the racemization (Kenyon and Hegeman, 1970). Positive kinetic ρ values can be expected neither for reactions which involve attack by a negatively charged nucleophile on the α-carbon in the transition state nor, of course, for reactions which involve accumulation of positive charge on the α-carbon in the transition state, such as in a mechanism involving a carbonium ion intermediate (Jaffé, 1953). Representative kinetic ρ values from the literature for some relevant reactions which do not involve enzymes are shown in Table VI. For substituted toluenes a negative ρ value was found for the radical hydrogen abstraction process, whereas a value of $\rho = +4.0$ was found for the reaction of the substituted toluenes involving a carbanion intermediate. In the case of the generation of the fluorene anion, which is stabilized by a greater degree of electron delocalization than the isolated toluene anion, the observed ρ value was only +3.2. Thus, if a carbanion intermediate be proposed for the mandelic acid racemase reaction, some mode of charge dispersal independent of stabilization due to the substituted phenyl group would have to be invoked to account for the lower ρ value observed.

The occurrence of a primary deuterium isotope effect is consistent with a mechanism involving either a proton, hydrogen atom, or hydride ion transfer process. The enhancement of the V_{max} observed for the racemization of the mandelic acids substituted with electron-withdrawing substituents would favor a proton transfer proceeding to a carbanion intermediate, but because of the difficulties in applying Hammett ρ–σ relationships to enzyme systems (Caplow and Jencks, 1962) we felt that more definitive answers to mechanistic questions might be derived from isotopic exchange studies. The results of these studies and our conclusions about the mechanism of the mandelic acid racemase reaction are presented in the following paper of this series (Kenyon and Hegeman, 1970).

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Mandelic Acid Racemase from *Pseudomonas putida*. Evidence Favoring a Carbanion Intermediate in the Mechanism of Action*

George L. Kenyon and George D. Hegeman

ABSTRACT: Substantial deuterium exchange occurs in the α-carbon position of mandelic acid during the racemization of unlabeled D-(−)-mandelic acid by mandelic acid racemase (EC 5.1.2.2) in 96% D_2O, or when the racemization of D-(−)-α-deuteriomandelic acid is catalyzed by the enzyme in H_2O. Under similar conditions no significant amount of ^{18}O was found to be incorporated into the mandelic acid when D-(−)-mandelic acid was enzymatically racemized in ^{18}O-enriched water. Benzoylformic acid was excluded as a reaction intermediate since, in the presence of the enzyme, [^{14}C]benzoylformic acid did not give rise to [^{14}C]mandelic acid and [^{14}C]mandelic acid did not yield [^{14}C]benzoylformic acid.

The enzyme catalyzed with approximately equal efficiency the incorporation of radioactivity into the D-(−) and L-(+) enantiomers during partial racemization of the D-(−)-mandelic acid in tritiated water. The V_{max} for the enzyme-catalyzed conversion of D-(−)-α-deuteriomandelic acid to the L-(+) enantiomer was approximately five times less than that observed for the normal protonated substrate. A mechanism involving a carbanion intermediate is consistent with these and other findings.

The apparent lack of pyridine nucleotide or flavin cofactors necessary for activity of mandelic acid racemase and the increase in rate accompanying substitution of the substrate in the 4 position by electron-withdrawing substituents (Hegeman *et al.*, 1970) suggest the occurrence of certain possible intermediates in the course of racemization mediated by the enzyme. In order to determine which intermediate or intermediates may be involved in the racemization process we have used various isotopes to investigate the possible occurrence of enzyme-catalyzed exchange between (1) the substrate and the solvent and (2) the substrate and the potential keto intermediate, benzoylformic acid.

Materials and Methods

Analytical and preparative gas–liquid chromatography was performed with an F&M Model 700 instrument using 0.125 or 0.25 in. × 6 ft columns packed with 10% diethylene glycol succinate on Chromosorb W (Applied Sciences Laboratories, Inc.). The chromatograph was fitted with a 1:1 stream splitter and flame ionization detector. Helium was employed as carrier gas (flow rate 40–60 ml/min) and the oven temperature was maintained at 150°. Samples were collected using glass U-tubes cooled in a Dry Ice–acetone bath.

Peak areas were integrated by weighing peaks excised from xerographic copies of the original chromatographic tracings.

Radioactivity was determined using a Nuclear Chicago, Inc. Model Mark I scintillation spectrometer. A toluene scintillation fluid containing 15.1 g of 2,5-diphenyloxazole and 0.189 g of *p*-bis[2-(5-phenyloxazolyl)]benzene per 3.78 l. of toluene was used.

A Varian Instruments Model T-60 spectrometer was used to determine nmr spectra.

Consolidated Electrodynamics Corp. Model 21-110B double-focusing high resolution mass spectrometer was used to determine mass spectra and make mass measurements.

D-(−)-Mandelic acid, DL-mandelic acid, and benzoylformic acid were obtained from the Aldrich Chemical Co. Diazomethane was generated using Diazald from the same company.

DL-[^{14}C]Mandelic acid, labeled in the carboxylate position, was prepared from benzaldehyde and [^{14}C]NaCN by the procedure of Fieser (1957) except that the sodium cyanide was used as the limiting reagent. Also, the crystals of the sodium bisulfite addition complex with benzaldehyde were isolated and purified by filtering and washing with dilute $NaHSO_3$ solution. The DL-[^{14}C]mandelic acid was purified

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