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Mandelate Racemase from *Pseudomonas putida.* Absence of Detectable Intermolecular Proton Transfer Accompanying Racemization[†]

Thomas R. Sharp,[‡] George D. Hegeman,* and George L. Kenyon[§]

ABSTRACT: An equimolar mixture of DL-[α-^{2}H]- and DL-[α-^{13}C]mandelate, when incubated with mandelate racemase (EC 5.1.2.2), shows conversion of singly labeled mandelate to unlabeled mandelate, due to solvent exchange of the α proton, while the level of doubly labeled mandelate remains at a constant low level. Similarly, an equimolar mixture of unlabeled and DL-[α-^{2}H,α-^{13}C]mandelate, when incubated with the enzyme, shows conversion of doubly labeled mandelate to singly labeled mandelate, due to solvent exchange, while the level of unlabeled mandelate remains constant at 50%. Incubation of an equimolar mixture of DL-[α-^{3}H]mandelate and DL-*p*-chloromandelate, both with similar properties as substrates for mandelate racemase, showed solvent exchange of the α-^{3}H of mandelate, but no ^{3}H appeared in the *p*-chloromandelate. These results indicate that mandelate racemase does not catalyze an intermolecular proton transfer to achieve racemization. These data are necessary, but not sufficient, results to indicate that mandelate racemase operates via a one-acceptor mechanism, in which the proton abstracted from one stereochemical face of a substrate molecule is returned to the opposite face of the same carbon of the substrate molecule.

Mandelate racemase (EC 5.1.2.2), an inducible enzyme isolated from *Pseudomonas putida* A.3.12 (ATCC 12633) grown at the expense of DL-mandelic acid or DL-*p*-hydroxymandelic acid (Gunsalus et al., 1953), has been shown to catalyze the racemization of mandelic acid without requiring an organic coenzyme (Hegeman et al., 1970). A divalent metal ion is required for activity, of which Mg^{2+} or Mn^{2+} (Fee et al., 1974) work best. Magnetic resonance studies (Maggio et al., 1975) have indicated that the metal ion assists in polarizing the carbon–hydrogen bond in order to promote abstraction of the α hydrogen as a proton. The enzyme has been shown to catalyze solvent exchange of the α proton concurrent with racemization and at a rate approximately 20% of the rate of racemization (Kenyon and Hegeman, 1970).

A primary deuterium isotope effect of approximately 5 has been observed (Kenyon and Hegeman, 1970) when the α proton is replaced with deuterium. Study of the reaction kinetics of a series of para-substituted mandelic acids as substrates for the enzyme has yielded results which suggest, when

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interpreted according to the Hammett ρ–σ relationship, that racemization proceeds through a carbanion intermediate (Hegeman et al., 1970). These facts show that one or more side chain groups of amino acid residue(s) in the protein must act as acceptors in removal of a proton from one stereochemical face of the substrate and replacement of a proton on the other face.

Rose (1966, 1970) has indicated that enzymes which catalyze hydrogen transfer without the assistance of an organic coenzyme and with at least partial concurrent solvent exchange may proceed by either a one- or two-acceptor mechanism. The ribulose-5-phosphate 4-epimerase reaction has been proposed to follow a one-acceptor mechanism (Davis et al., 1972). Both proline racemase (Cardinale and Abeles, 1968; Rudnick and Abeles, 1975) and hydroxyproline 2-epimerase (Finlay and Adams, 1970) have been shown to follow two-acceptor mechanisms, both acceptors in both enzymes being identified as cysteine sulfhydryl groups.

This report will present the results of isotope-exchange experiments, which indicate that mandelate racemase does not catalyze an intermolecular proton transfer to achieve racemization and that these are necessary, but not sufficient, results to indicate that mandelate racemase operates via a one-acceptor mechanism in which a single nucleophilic amino acid residue side chain abstracts the α proton from one face of a mandelate molecule and returns the same proton to the same carbon skeleton to produce the opposite enantiomer.

Experimental Procedures

Materials. Mandelate racemase was prepared according to the method of Hegeman (1970) from *Pseudomonas putida* A.3.12 (ATCC 12633). Cultures of this organism were grown on Hutner's modified mineral medium with ammonium DL-mandelate as sole carbon and energy source. Enzyme purity was assessed by polyacrylamide gel electrophoresis (both nondenaturing and denaturing conditions), and the enzyme was found to be better than 95% pure.

Authentic D- and L-mandelic acids were purchased from Sigma Chemical Co. DL-Mandelic acid and DL-*p*-chloromandelic acid were obtained from Aldrich Chemical Co. DL-[α-^{13}C]Mandelic acid was prepared by Dr. E. T. Maggio (Maggio et al., 1975). DL-[1-^{14}C]Mandelic acid was prepared as previously described (Kenyon and Hegeman, 1970). DL-[α-^{3}H]Mandelic acid was prepared by dissolving a portion of DL-mandelic acid in tritium-enriched water (ICN biological grade, lot #994573, final specific activity 10 mCi/mL) made 6 N in HCl and heated at 110 °C for 19 h. The mandelate was isolated by taking the solution to dryness over Drierite and NaOH in vacuo. The sample was dissolved in H_2O and dried in vacuo three times to remove freely exchangeable tritium.

Both DL-[α-^{2}H]- and DL-[α-^{2}H,α-^{13}C]mandelic acids were prepared from the appropriate starting materials by incubation in 2 M KOH in 99.5% D_2O (Bio-Rad Laboratories) at 110 °C for 24 h. The mandelates were reisolated by acidification of the solutions to below pH 1 with concentrated HCl, extraction three times with equal volumes of ether, and the ether was evaporated in a stream of dry N_2. The samples were dissolved three times in neutral buffer solution, acidified, and extracted in order to replace the freely exchangeable deuterium with hydrogen.

Previous studies have shown by NMR[1] (Kenyon and Hegeman, 1970) and by mass spectral isotope-retention patterns (Sharp, submitted for publication) that incorporation of deuterium or tritium into positions other than the α, hydroxyl, or carboxyl positions under acid- or base-catalyzed solvent exchange is negligible.

Methods. When samples of reaction mixtures were removed during incubation, the reaction was quenched by addition to at least an equal volume of 1 N HCl, and the sample was immediately extracted three times with equal volumes of ether. The combined ethereal extracts were dried over anhydrous $MgSO_4$ and the ether evaporated in a stream of dry N_2.

Dried samples were submitted for mass spectrometric analysis without further treatment. Mass spectrometric measurements were conducted on the Varian MAT CH-7 mass spectrometer located in the mass spectrometry laboratories of the Department of Chemistry, Indiana University, Bloomington, Ind.[2] Samples of mandelic acid were introduced into the ion source by the direct probe inlet; the probe typically required warming to 40–50 °C. Electron-impact ionization was used (70 eV). Repeated slow scans of the base peak (*m/e* 107) and molecular ion (*m/e* 152) regions of the mass spectrum of each sample were recorded. Peak heights of the fragments occurring at *m/e* 107, 108, and 109, and *m/e* 152, 153, and 154 were measured for subsequent calculations. This approach to measurement of isotopic content of molecules which have been artificially enriched has been successfully employed in the past (Swain et al., 1963).

Intensities of the fragment peaks at *m/e* 107, 108, and 109, and at *m/e* 152, 153, and 154 were taken to be representative of the relative abundances of unlabeled, singly labeled, and doubly labeled species, respectively, in samples taken from incubation mixtures. Percentage of total for a particular labeled species was calculated by dividing the intensity of a given peak by the sum of the three peak intensities in that region—e.g., $\%107 = 107/(107 + 108 + 109) \times 100$ (mass numbers represent the intensities occurring at that mass). We have routinely observed precisions for these measurements of $\leq 2.0\%$.

In the experiments requiring purification of substrates for radioactive analysis, the dried samples were methyl esterified by addition of an ethereal solution of diazomethane until an excess of the derivatizing agent was indicated by residual yellow color. Diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald, Aldrich Chemical Co.) according to the method described in Fieser and Fieser (1967). Excess diazomethane and ether were evaporated in a stream of dry N_2, and the residue was redissolved in a minimal volume of $CHCl_3$.

The $CHCl_3$ solutions of methyl esters of the substrates were chromatographed using a Hewlett-Packard F&M, Model 402, high-efficiency gas chromatograph equipped with flame ionization detector (FID) and a ~10:1 stream splitter. The column packing used was 10% diethylene glycol succinate (DEGS) on Chromosorb w, 80–100 mesh (Applied Sciences Labs, Inc.), in a 2 mm × 2 m glass U-tube column. A temperature of 170 °C and helium carrier gas flow rate of 50 mL/min were found

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[1] Abbreviations used are: FID, flame ionization detector; DEGS, diethylene glycol succinate; NMR, nuclear magnetic resonance; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; E, $E{<_H}$ and $E{<^H}$, free enzyme forms, the latter two representing a two-acceptor enzyme with different proton acceptors in the conjugate acid form; LM and DM, L- and D-mandelate, respectively; E·LM, E·DM, $E{<^H}$·LM, and $E{<_H}$·DM, the appropriate Michaelis complexes; EH·M and EH*·M, proposed transition states in which the α proton has been transferred to the enzyme, with the M representing the substrate carbon skeleton, and the H* representing a proton which has come from the solvent.

[2] Development of these facilities was made possible by funds supplied to the Department of Chemistry, Indiana University, by National Science Foundation Grant GP-32225.

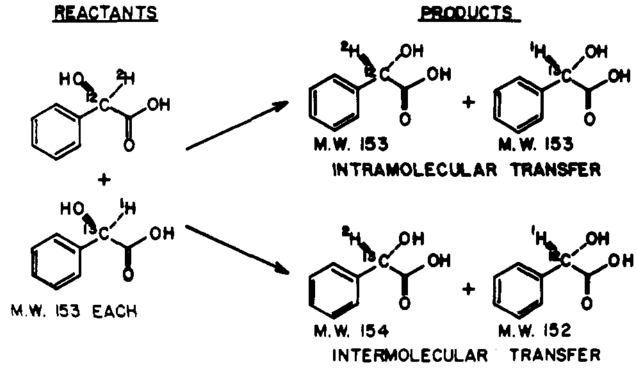
to give satisfactory resolution of the methyl mandelate and methyl *p*-chloromandelate fractions and convenient retention times (methyl mandelate, 4.5 min; methyl *p*-chloromandelate, 10.5 min) for the manipulations necessary.

Collection of fractions for measurement of radioactivity was achieved by placing a Teflon tube over the outlet of the larger fraction side of the stream splitter as the desired fraction was eluted (as indicated by the mass trace from the FID connected to the smaller fraction side of the stream splitter), with a loop of the Teflon tube immersed in a Dewar flask containing an acetone–dry-ice mixture. After the desired fraction had eluted from the chromatograph, the Teflon tube was removed, and the contents of the tube were washed into a glass scintillation vial with 10 mL of toluene scintillation fluid (4 g of PPO and 0.05 g of POPOP in 1 L of toluene). Samples were counted at least twice for 10 min in a Beckman LS-233 liquid scintillation counter against an external standard, using the standard ^{3}H and ^{14}C window settings supplied with the instrument. ^{3}H and ^{14}C counts per min were corrected to disintegrations per min using a FORTRAN computer program (Sharp, unpublished), which corrects for variable counting efficiency and count spillover. Subsequent calculations were carried out using the disintegrations per minute values calculated by this program.

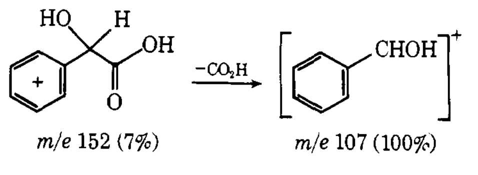
Results

[α-^{2}H]Mandelate–[α-^{13}C]Mandelate Exchange. Depending upon the number of proton acceptors in the active site of mandelate racemase, the products expected from incubation of an equimolar mixture of DL-[α-^{2}H]mandelate and DL-[α-^{13}C]mandelate in the presence of the enzyme are indicated in Scheme I. If the enzyme contains two proton acceptors in the active site, then intermolecular proton transfer would be expected, producing a doubly labeled species (carrying both α-^{2}H and α-^{13}C labels) and an unlabeled mandelate molecule. The presence of a single proton acceptor would require conservation of the α-deuterium in the same substrate molecule and would not permit production of a doubly labeled species.

Scheme I



The first step in the fragmentation of mandelic acid in the mass spectrometer is loss of 45 mass units from the molecular ion (*m/e* 152) by loss of CO_2H, yielding the base peak at *m/e* 107. Isotope retention patterns in the mass spectra of the pure [α-^{2}H]- and [α-^{13}C]mandelic acids have shown (Sharp, submitted for publication) that both molecular ion and base-peak ion carry the α position in which the ^{2}H and ^{13}C labels of interest in this experiment reside. Moreover, the elemental compositions of both the parent ion at *m/e* 152 and the base peak at *m/e* 107 have been confirmed by accurate mass measurements (Kenyon and Hegeman, 1970).



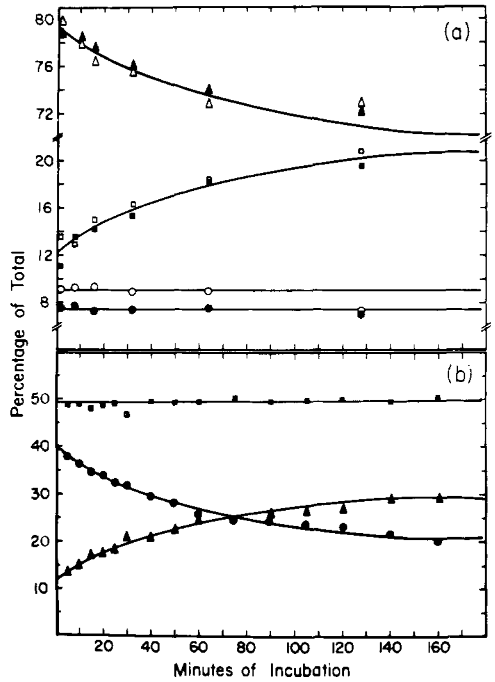


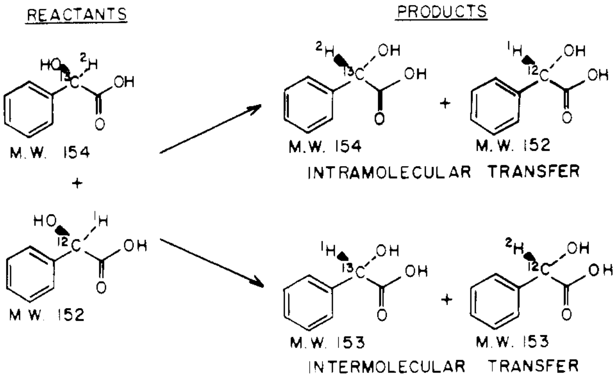
FIGURE 1: Time courses for mass label exchange experiments. (a) Depicts DL-[α-^{2}H]mandelate–DL-[α-^{13}C]mandelate exchange, with an equimolar mixture of each labeled species, 0.1 M total mandelate as the sodium salts; (b) depicts DL-[α-^{2}H,α-^{13}C]mandelate–DL-mandelate exchange, with an equimolar mixture of each labeled species, 0.02 M total mandelate as the Na$^+$ salts. Both reactions were incubated at 25 °C in 0.1 M sodium phosphate buffer, pH 7.5, with 0.1 mg/mL protein. In both panels, % *m/e* 152 (□) and % *m/e* 107 (■) represent unlabeled, % *m/e* 153 (△) and % *m/e* 108 (trus) represent singly labeled mandelate, and % *m/e* 154 (○) and % *m/e* 109 (●) represent doubly labeled mandelate.

For a two-acceptor mechanism, the percentage of doubly labeled and unlabeled species should increase at the expense of singly labeled species. For a one-acceptor mechanism, no change in the percentages of each species should be seen. Since mandelate racemase catalyzes a partial solvent exchange at a rate of approximately 20% of the rate of racemization (Kenyon and Hegeman, 1970), this fact changes the above predicted results in the following manner. A doubly labeled species should increase transiently, then decrease due to solvent exhange for a two-acceptor model. For a one-acceptor model, a doubly labeled species would stay at a constant low level (due to natural abundance ^{13}C in other parts of the substrate molecule), while an unlabeled species should increase at the expense of a singly labeled species, due to solvent exchange of the α-^{2}H. Figure 1a shows the percent of total for the various labeled species over the time course of incubation with mandelate racemase, as indicated by both the base peak and molecular ion regions of the mass spectrum. The total mandelate concentration for this experiment was 0.1 M.

[α-^{2}H,α-^{13}C]Mandelate–Mandelate Exchange. Scheme II outlines a second experiment in which DL-[α-^{2}H,α-^{13}C]-mandelate was incubated with mandelate racemase in an equimolar mixture containing both this doubly labeled mandelate and unlabeled mandelate (0.02 M total mandelate). For a two-acceptor mechanism, the enzyme would be expected to

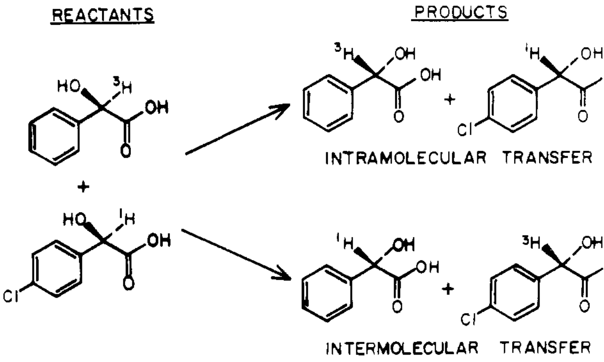
catalyze an intermolecular transfer, transiently producing singly labeled species at the expense of *both* unlabeled and doubly labeled species. A one-acceptor mechanism would cause only solvent exchange, converting the doubly labeled species to a singly labeled species, while not affecting the level of unlabeled species. Figure 1b shows the results of this experiment. Data plotted are for the base peak ion. Peak intensities in the molecular ion region were analyzed (but are not shown) and gave the same behavior with time.

Scheme II



[α-^3H]*Mandelate-p-Chloromandelate Exchange.* According to Scheme III, if an equimolar mixture of DL-p-chloromandelate and DL-[α-^{3}H]mandelate were incubated in the presence of mandelate racemase, tritium would be transferred from mandelate to p-chloromandelate if the two-acceptor model described above was being followed, and at least a transient appearance of tritium activity in p-chloromandelate would be expected along with disappearance of tritium from mandelate. If the one-acceptor model were being followed, tritium solvent exchange should occur, but no tritium should appear in the p-chloromandelate.

Scheme III



Because of the inherent imprecision of measurements of amounts of mandelate or p-chloromandelate based upon calibration curves for the FID of the gas chromatograph, tritium specific activities could not be reliably calculated for samples containing only tritium. Changes in the tritium specific activity of mandelate were therefore measured by including in the original substrate mixture a small amount of DL-[1-^{14}C]-mandelate as an internal standard. Since the catalytic action of mandelate racemase does not alter the carbon skeleton of the substrate, the ^{14}C specific activity of mandelate remains constant throughout the experiment, and changes in the ^{3}H specific activity of mandelate could be more sensitively measured by a change in the ^{3}H/^{14}C activity ratio.

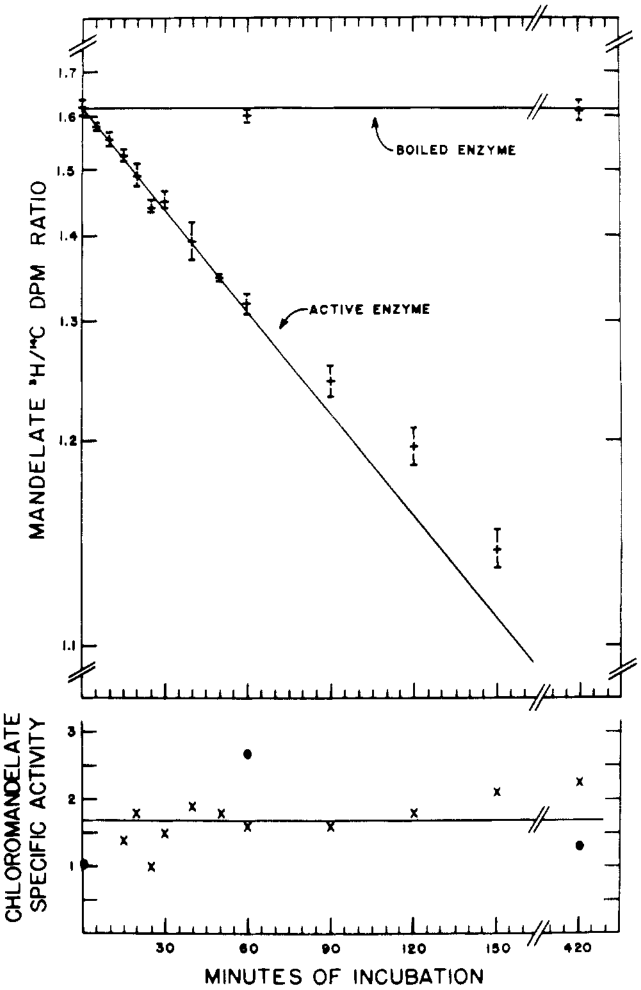


FIGURE 2: Specific activity time course of methyl mandelate and methyl p-chloromandelate derived from the DL-[α-^{3}H]mandelate-p-chloromandelate exchange. Incubation was conducted at 25 °C in 0.1 M sodium phosphate buffer, pH 7.5, with 0.05 mg/mL protein and 0.02 M of each substrate (Na$^+$ salts). Error bars on ^{3}H/^{14}C points indicate plus or minus one standard deviation. A sample of enzyme was boiled for 10 min prior to mixing with substrate in the control. Protein concentrations were equal in the control and experimental incubations. Experimental (x) and boiled enzyme control (●) samples are indicated on the methyl p-chloromandelate specific activity plot.

Because ^{14}C-labeled p-chloromandelate was not available, the tritium specific activities for p-chloromandelate are expressed in arbitrary units consisting of the total tritium activity observed in a collected sample divided by the peak height recorded by the FID for that sample. All p-chloromandelate peaks were recorded at a constant attenuator setting on the gas chromatograph. Figure 2 shows the results for this experiment.

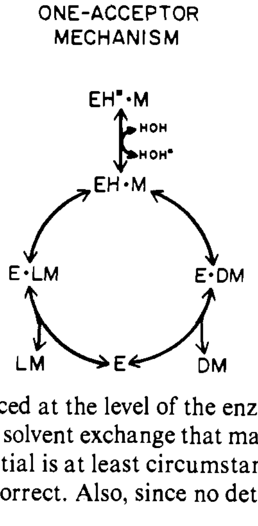
Discussion

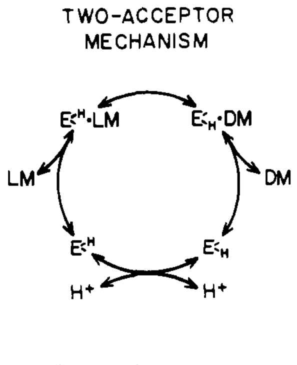
Of the general class of isomerases, epimerases, and racemases which do not require an organic coenzyme for catalytic activity, most of the well-characterized examples appear to follow a two-acceptor type of catalytic mechanism, in which two distinct amino acid residues mediate the removal of a hydrogen from one stereochemical face of the substrate and place a *different* hydrogen on the opposite face. Rose (1966, 1970) has discussed in depth the concepts of the one-acceptor–two-acceptor dichotomy for this group of enzymes, and Adams (1976) summarized the data supporting the above statement in Table IX of his recent extensive review.

Proline racemase (Cardinale and Abeles, 1968) and hydroxyproline 2-epimerase (Finlay and Adams, 1970) have been shown to catalyze deuterium incorporation from D_2O solvent into the product enantiomer from optically pure starting material at a rate equal to that of racemization. Similar results have also been reported for D-ribulose-5-phosphate 3-epimerase (Davis et al., 1972). These results imply that one nucleophile in the enzyme active site abstracts the substrate hydrogen while a second nucleophile places a different hydrogen, derived from the solvent, onto the substrate. Furthermore, rates of tritium solvent exchange of [^{3}H]proline by proline racemase (Rudnick and Abeles, 1975) under both initial-velocity and equilibrium conditions as a function of substrate concentration indicate that solvent exchange can occur only after product has dissociated from the enzyme. These results lead to the conclusion that two microscopically distinct enzyme forms exist, each of which can productively bind only one proline enantiomer, and which differ only by the identity of the active-site acceptor which is in its conjugate acid form.

Conversely, partial or total hydrogen retention upon enzymatic transformation has been reported for some enzymes of this class. Δ^5-3-Ketosteroid isomerase (Wang et al., 1963), lactate racemase (Shapiro and Dennis, 1965; Hiyama et al., 1968), and L-ribulose-5-phosphate 4-epimerase (Davis et al., 1972) show total hydrogen retention, while aconitase (Rose and O'Connell, 1967), glucose-6-phosphate isomerase (Rose and O'Connell, 1961), and mandelate racemase show partial retention of the hydrogen in question. All these enzymes (excepting lactate racemase, which has been proposed to follow a hydride transfer mechanism; Shapiro and Dennis, 1965; Cantwell and Dennis, 1974) have had a single nucleophilic amino acid residue side chain implicated in transfer of the hydrogen as a proton. In addition, all but mandelate racemase catalyze the net migration of the proton from one carbon atom to another in the substrate through an enediol intermediate. Mandelate racemase is thus far the only enzyme in this class for which the reported data indicate that a proton is removed from one carbon and placed back onto the same carbon to yield the opposite enantiomer.

In order to achieve this type of transfer, two possible schemes might be proposed for the catalytic mechanism of mandelate racemase. The schemes for a one- and a two-acceptor mechanism, between which we have set out to distinguish, are presented below, the latter being modeled after that of Rudnick and Abeles (1975). (The symbols used are defined in the abbreviations footnote.[1]) It should be pointed out that, in the one-acceptor scheme, the site of solvent exchange is tentatively placed at the level of the enzyme–substrate intermediate. That the solvent exchange that mandelate racemase catalyzes is only partial is at least circumstantial evidence that this placement is correct. Also, since no detectable carbon skeleton exchange was observed when mandelate racemase was incubated with unlabeled mandelate and [^{14}C]benzoylformate (a potential intermediate; Kenyon and Hegeman, 1970), this fact eliminates a route analogous to that of aconitase (Rose and O'Connell, 1967), in which solvent exchange can be achieved by dissociation of the intermediate stable compound, *cis*-aconitate.





For the experiment with DL-[α-^{2}H]- and DL-[α-^{13}C]-mandelates as starting materials (Figure 1a), a one-acceptor mechanism with concurrent partial solvent exchange leads to the prediction of an increase in unlabeled mandelate at the expense of singly labeled mandelate while doubly labeled mandelate remains constant. For the experiment with DL-[α-^{2}H,α-^{13}C]mandelate and unlabeled mandelate as starting materials (Figure 1b), the conversion of doubly labeled mandelate to singly labeled mandelate, while the unlabeled mandelate remains constant, is predicted. The experimental results reported above consistently agree with what would be expected, should a one-acceptor mechanism be operating in mandelate racemase.

Yet another experimental approach is to look for intermolecular proton transfer between two similar substrates in the same incubation mixture. Of the several para-substituted mandelic acid analogues previously examined (Hegeman et al., 1970), *p*-chloromandelate shows a K_M nearly equal to that of mandelate (100 μM for *p*-chloromandelate vs. 93 μM for mandelate), while the V_{max} of *p*-chloromandelate was approximately threefold higher than that for mandelate (107.5 μmol/min vs. 33 μmol/min).

Mandelate racemase catalyzes a pseudo-first-order tritium exchange from the mandelate, but, within experimental error, the tritium does not appear in the *p*-chloromandelate. The smooth pseudo-first-order plot for exchange of tritium (Figure 2) for a DL mixture supports the previous contention (Maggio et al., 1975) that the enzyme catalyzes racemization (and solvent exchange) of D-mandelate at the same rate as that of L-mandelate.

Some tritium activity was observed in the *p*-chloromandelate collections, but this observation is not considered significant for several reasons. First, as is shown in Figure 2, the *p*-chloromandelate specific activity remains essentially constant over the incubation period. The scatter of points is due to the imprecision of calculating specific activities based upon FID peak heights. Also, the apparent upward trend in *p*-chloromandelate specific activity starting at 120-min incubation is an artifact of the plotting process. An equal number of data points extending out to 2160 min (36 h) of incubation lies on or below the drawn line.

Second, comparison of the absolute numbers of tritium counts occurring in a given *p*-chloromandelate collection and its corresponding mandelate fraction shows that less than 10%, and, more typically, 1–5% of the total tritium activity injected onto the gas chromatographic column appeared in the *p*-chloromandelate fraction. Finally, the tritium activity appearing in the *p*-chloromandelate fraction was always accompanied by more than enough ^{14}C activity—the only source of which in this experiment was [1-^{14}C]mandelate—to indicate that tritium activity appearing in the *p*-chloromandelate fraction was a result of contaminating mandelate. Support for these ideas comes from the observation of the same degree of scatter for tritium specific activities and ^{14}C contamination found in the *p*-chloromandelate fractions derived from the boiled enzyme control (see Figure 2).

Again, the results of this experiment indicate the absence of intermolecular proton transfer during racemization, and support the one-acceptor model for the catalytic mechanism of mandelate racemase. Note also that the control for exchange of mandelate itself using boiled enzyme (see Figure 2) confirms the earlier finding (Kenyon and Hegeman, 1970) that only active enzyme promotes solvent exchange of the α proton.

Experiments similar to those described above have been reported for other enzymes, with the same goal intended. Unlabeled L-ribulose 5-phosphate and D-[1,4,6,6-^{2}H]xylulose 5-phosphate were incubated with L-ribulose-5-phosphate 4-epimerase (Davis et al., 1972) with no observed intermolecular transfer of the 4-deuterium and no solvent exchange. Malhotra and Ringold (1965) have performed experiments with Δ^5-3-ketosteroid isomerase, both with mixed substrates and with doubly labeled substrates, and observed no intermolecular hydrogen transfer in either instance.

The two mechanisms shown above are the simplest schemes consistent with data available prior to this report. Moreover, the experiments performed here cannot distinguish between the one-acceptor mechanism and a two-acceptor mechanism in which rapid direct proton transfer between the two acceptors occurs. Experimental data which suggest this latter mechanism, however, have not appeared in the literature. Davis et al. (1972) discussed this "conducted tour" type of mechanism in the context of their findings with L-ribulose-5-phosphate 4-epimerase and discounted it. They could not induce solvent exchange with this enzyme even in the presence of 7.5 M urea (the enzyme remains 30% active under these conditions). These conditions might have been expected to expose a pair of proton acceptors in the active site at least partially to the solvent, if they indeed existed.

That an enzyme that normally catalyzes intramolecular hydrogen transfer can be induced to catalyze intermolecular transfer by adjusting the incubation conditions is exemplified by the situations with aconitase and fumarase. When tritiated citrate, isocitrate, 2-methylcitrate, or 2-methylisocitrate were incubated with aconitase with no added *cis*-aconitate, typical intramolecular tritium transfer was observed (Rose and O'Connell, 1967). When tritiated 2-methylcitrate or 2-methylisocitrate were incubated with aconitase at high added *cis*-aconitate, tritium was transferred from the 2-methyl substrates to the *cis*-aconitate carbon skeleton, producing tritiated citrate and isocitrate. Similarly, fumarase could be forced to perform intermolecular tritium transfers between carbon skeletons at high fumarate concentration and decreased water concentration (1:1 water-glycerol solvent; Hansen et al., 1969), as indicated by a malate-fumarate carbon skeleton exchange rate being faster than the tritium exchange rate between [^{3}H]-malate and water. We have attempted to induce mandelate racemase to catalyze intermolecular hydrogen transfer by conducting the [α-^{2}H]mandelate-[α-^{13}C]mandelate exchange at 0.1 M total substrate (compared with a K_M for mandelate of 93 μM; Hegeman et al., 1970). The results quite clearly indicated a lack of intermolecular transfer (Figure 1a).

Conclusions

All three sets of experiments presented here are in agreement in that no detectable intermolecular proton transfer has been observed. The facts that mandelate racemase does not appear to catalyze intermolecular proton transfer, and that only approximately 20% solvent exchange of the α-proton occurs, lead to the prediction that mandelate racemase catalyzes an *intramolecular* proton transfer, returning the proton abstracted from one face of a substrate molecule to the opposite face of the same molecule. Very recent preliminary evidence confirms this prediction (Sharp et al., 1976), in that D-[α-^{3}H]mandelate has been found to be formed from L-[α-^{3}H]mandelate during racemization promoted by mandelate racemase.

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