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Mechanism of the Reaction Catalyzed by Mandelate Racemase. 1. Chemical and Kinetic Evidence for a Two-Base Mechanism†

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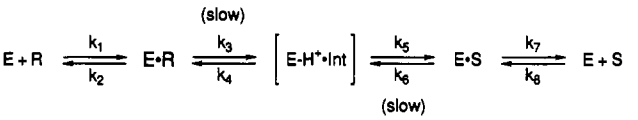
Received April 4, 1991; Revised Manuscript Received June 14, 1991

ABSTRACT: The fate of the α-hydrogen of mandelate in the reaction catalyzed by mandelate racemase has been investigated by a mass spectroscopic method. The method entails the incubation of (*R*)- or (*S*)-[α-^{1}H]mandelate in buffered D_2O to a low extent of turnover (about 5–8%), esterification of the resulting mixture of mandelates with diazomethane, derivatization of the methyl esters with a chiral derivatizing agent, and quantitation of the isotope content of the α-hydrogen of both substrate and product by gas chromatography/mass spectrometric analysis. No significant substrate-derived α-protium was found in the product for racemization in either direction. In addition, in the (*R*) to (*S*) direction almost no exchange (≤0.4%) of the α-hydrogen in the remaining (*R*) substrate pool occurred, but in the (*S*) to (*R*) direction 3.5–5.1% exchange of the α-hydrogen in the remaining substrate (after 5.1–7.2% net turnover) was found. Qualitatively similar results were obtained in the (*S*) to (*R*) direction in H_2O when (*S*)-[α-^{2}H]mandelate was used as substrate. In other experiments, an overshoot in the progress curve was observed when the racemization of either enantiomer of [α-^{1}H]mandelate in D_2O was monitored by following the change in ellipticity of the reaction mixture; the magnitude of the overshoot was greater in the (*R*) to (*S*) than in the (*S*) to (*R*) direction. All of the available data indicate that the reaction catalyzed by mandelate racemase proceeds by a two-base mechanism, in contrast to earlier proposals.

Mandelate racemase (MR)[1] (EC 5.1.2.2) from *Pseudomonas putida* ATCC 12633 catalyzes the interconversion of the (*R*) and (*S*) enantiomers of mandelic acid via abstraction of the α-hydrogen as a proton. Catalysis is thought to involve an intermediate that has at least partial resonance-stabilized carbanionic character[2] on the basis of the effect of electron withdrawing/donating groups on the velocity of the reaction

Scheme I



and the elimination of halide ions from *p*-(halomethyl)-mandelates (Kenyon & Hegeman, 1979; Lin et al., 1988,

† This work was supported by Grants GM-40570 (J.A.G., J.W.K., and G.L.K.), AR-17323 (G.L.K.), GM-34572 (J.A.G.), and GM-37210 (J.W.K.). V.M.P. was supported by a predoctoral fellowship from the American Foundation for Pharmaceutical Education, and C.W.K. was supported by a National Institutes of Health Predoctoral Traineeship. This is paper 13 in a series on mandelate racemase; paper 12 is Neidhart et al. (1990).
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[1] Abbreviations: CD, circular dichroism; FID, flame ionization detector; GC, gas chromatograph(y); H297N, the mutant of mandelate racemase in which histidine 297 has been replaced with asparagine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MR, mandelate racemase; MTPA, α-methoxy-α-(trifluoromethyl)phenylacetic acid; MW, molecular weight; NMR, nuclear magnetic resonance spectroscopy; PLP, pyridoxal phosphate.

0006-2960/91/0430-9255$02.50/0

1991); this intermediate presumably is stabilized by association of the carboxylate group with both the required Mg^{2+} and the ϵ-ammonium group of Lys 164 (Maggio et al., 1975; Neidhart et al., 1991). The kinetics of the reaction are highly symmetrical in spite of the inherent chirality of the enzyme (Whitman et al., 1985). The virtually identical kinetic constants (±15%) measured for the two enantiomers and the moderately large primary deuterium kinetic isotope effects (3.2 and 3.6 for (*R*)- and (*S*)-mandelate, respectively) have led to the working model for the kinetic mechanism shown in Scheme I. The partially rate-determining formation of the intermediate ($k_3 \sim k_6$) is followed by the equi-partitioning of the intermediate between the forward and backward directions ($k_4 \sim k_5$). The chemical details of the catalytic mechanism, however, have been unclear.

In contrast to isomerases, which usually involve suprafacial proton transfers, racemases and epimerases that proceed by putative intermediates must effect the net transfer of a proton from one side of the intermediate to the other to accomplish the net inversion of configuration of the carbon atom. The mechanisms of these enzymes have usually been evaluated in terms of the one-acceptor (one-base) and two-acceptor (two-base) mechanisms [for reviews, see Rose (1966), Cardinale and Abeles (1968), Adams (1976), and Soda et al. (1986)]. In the one-base mechanism, an enzyme base abstracts the substrate proton, generating the intermediate, and then reprotonates either side stereorandomly to give either substrate or product. In the two-base mechanism, the abstraction and protonation steps are effected by two enantiomer-specific bases juxtaposed on either side of the chiral carbon. One base abstracts the proton from the substrate enantiomer, and the conjugate acid of the second base protonates the intermediate from the other side to give the product enantiomer. These roles are reversed for racemization in the opposite direction. In principle, these mechanisms are distinguishable on the basis of the extent of substrate-derived proton exchange with solvent (Rose, 1966; Cardinale & Abeles, 1968). A one-base mechanism is indicated when a substantial amount of "internal return" (retention of the substrate-derived proton in the product) is observed or, given a moderate to negligible amount of internal return, when the ratio of the rates of solvent exchange into the product and substrate is the same regardless of which enantiomer is used as substrate. A two-base mechanism, on the other hand, precludes internal return, since the base that protonates the product side of the intermediate presumably acquires its proton from solvent. In addition, if the bases are monoprotic and do not exchange the substrate-derived proton at the stage of the intermediate, little or no solvent hydrogen should appear in the remaining substrate pool, regardless of substrate chirality. These general criteria have been used to infer two-base mechanisms for proline racemase (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975), hydroxyproline 2-epimerase (Finlay & Adams, 1970), diaminopimelic acid epimerase (Wiseman & Nichols, 1984), methylmalonyl-CoA epimerase (Leadley & Fuller, 1983; Fuller & Leadley, 1983), and ribulose-5-phosphate 3-epimerase (Davis et al., 1972). One-base mechanisms have been inferred for α-amino-ϵ-caprolactam racemase (Ahmed et al., 1986) and several alanine racemases (Faraci & Walsh, 1989, and references cited therein).

Previously, MR had been proposed to proceed by a one-base mechanism (Whitman et al., 1985; Kenyon & Hegeman, 1979). This viewpoint rested primarily upon unpublished studies that had indicated a substantial amount (~30%) of internal return using a tritium tracer label (Sharp, 1977). However, various limitations in the methodology employed at that time left some uncertainty as to the validity of the result. As part of a concerted program to determine the molecular mechanism for the reaction catalyzed by MR, we have reinvestigated the question of the number of bases that mediate proton transfer. In this paper we present evidence supporting a two-base mechanism. We find that not only are the hydrogen-exchange patterns consistent only with a two-base mechanism but also overshoots of differing magnitude are displayed in D_2O for the racemization of either [α-^{1}H]-mandelate enantiomer. In the two papers that follow (Neidhart et al., 1991; Landro et al., 1991) the atomic structure of the enzyme is revealed and the question of the identities of the two catalytic bases is addressed.

Materials and Methods

Highly purified MR was prepared according to the method of Tsou et al. (1989). The specific activity was 1050 units/mg ($k_{cat} \sim 700\ s^{-1}$ per 39000 MW monomer), as determined by use of the circular dichroic assay (Sharp et al., 1979) in 100 mM HEPES, pH 7.5, containing 5 mM (*R*)-mandelate and 3 mM $MgCl_2$, at 25 °C. Stock enzyme concentrations were determined by use of the method of Lowry et al. (1951) with bovine serum albumin (Sigma) as the standard. D_2O (99.8%) and all chemical starting materials were from Aldrich. (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA; "Mosher's acid") as well as (*R*)- and (*S*)-mandelates were all greater than 99% enantiomerically pure. Diazomethane was prepared as an ethanol-free ethereal solution from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) according to directions supplied. (*R*)- and (*S*)-MTPA acid chlorides (MTPA-Cl) were prepared from the respective free acids according to the method of Dale et al. (1969). Wheaton 1-mL teflon-capped Reacti-vials were from VWR Scientific.

Hydrogen Tracer Experiments in D_2O. The majority of the internal return experiments entailed the incubation of enzyme in buffered D_2O with (*R*)- or (*S*)-[α-^{1}H]mandelate until ~8% of the substrate had been converted to product. The extent of conversion of substrate to product was assessed for each aliquot by the derivatization and gas chromatography (GC) procedures described below. Aliquots in which substrate turnover had proceeded to about 5–8% completion were then subjected to GC/mass spectrometric analysis, and the isotopic compositions of both the product and the substrate were determined. A second set of experiments using (*S*)-[α-^{2}H]-mandelate as substrate in H_2O is described in a later paragraph.

Reactions (50 mL) were composed of 20 mM HEPES in D_2O, pD 7.5, containing 3 mM $MgCl_2$ and 5 mM (*R*)- or (*S*)-[α-^{1}H]mandelate. The reactions were equilibrated at 25 °C in a circulating water bath. After a 10-mL aliquot had been removed as a control, the reaction was initiated by the addition of 375 μL of solution of MR freshly prepared in buffered D_2O [prepared by the 1000-fold dilution of a concentrated stock solution (12000 units/mL) into reaction buffer]. At times of 4, 7, 11.5, and 16 min, 10-mL aliquots

[2] Whether the intermediate is an enolate anion (carbanion) derived by abstraction of a proton from mandelate anion or a charge-neutralized mandelate anion (protonated or metal ion coordinated) or an enol that could be obtained by protonation of an enolate anion is presently unknown. While this distinction is mechanistically important, the studies described in this and the following papers (Neidhart et al., 1991; Landro et al., 1991) address only the number and identities of the active site bases that are involved in forming the putative intermediate and not the extent of charge neutralization of the intermediate. Accordingly, the product of proton abstraction from mandelate by an active site base will be referred to as the "intermediate".

were removed. Each was quenched by adding 30 μL of 20% deuterium chloride (final pH = 2) and then quickly extracted with three 10-mL volumes of ether. [Control experiments showed that the acidification completely and immediately inactivated the enzyme while causing neither the introduction of deuterium into nor the racemization of the mandelates. Moreover, the enzyme was found to remain fully active for the duration of the incubations (Powers, 1989).] The combined ethereal layers of each aliquot were concentrated under reduced pressure. The deuteria at exchangeable sites were replaced with protia by dissolving each dried mandelate sample in 5 mL of H_2O, acidifying with 8 μL of 40% HCl, extracting with three 5 mL volumes of ether, drying over $MgSO_4$, removing the ether under reduced pressure, and repeating this exchange process once after dissolution in another 5-mL volume of water.

Diastereomeric Derivatization of the Mandelate Mixtures with (R)- or (S)-MTPA-Cl. The resulting mandelate samples were transferred to 1-mL Reacti-vials as ethereal solutions, and the ether was removed by nitrogen stream. The dried aliquots were methylated with diazomethane and then derivatized with either (*R*)- or (*S*)-MTPA-Cl as follows. To each dried aliquot (containing a maximum of 7.6 mg of mandelate; 50 μmol, 1 equiv) was added a slight excess of ethereal diazomethane (~1 mL) so that its yellow color remained after bubble evolution ceased. After 10 min the remaining ethereal diazomethane was removed by a nitrogen stream. To the resulting oil was added 250 μL of carbon tetrachloride, 250 μL of pyridine (dried over KOH), and 19 μL of MTPA-Cl (94 μmol, density = 1.25 g/mL, 1.9 equiv); the vial was capped, shaken briefly, and kept in darkness at room temperature for 20 h. Note that (*S*)-MTPA-Cl was used for incubations in which (*R*)-mandelate was the substrate, and (*R*)-MTPA-Cl was used when (*S*)-mandelate was substrate, so that the *product* mandelate derivative [either (*R*,*R*) or (*S*,*S*)] would elute cleanly from the GC column before the much larger, tailing substrate-derived peak. After 20 h, 400 μL of 0.5 M HCl was added, and the mixture was transferred to a 10-mL test tube containing 4 mL of ether. The ethereal layer was then extracted four times with 4-mL portions of 0.5 M HCl, three times with saturated sodium carbonate, and one time with water. After drying over $MgSO_4$, the ethereal solution was transferred to a new vial and concentrated to dryness with nitrogen. The products of the derivatization procedure were verified by ^1H NMR on control samples. No contaminants were present as judged both by NMR and by GC analysis.

Determinations of Extent of Turnover. To determine the extent of turnover, the derivatized mandelates from each incubation aliquot were base-line-resolved on a DB-5 capillary column (30 m, 0.32-mm i.d.; J & W Scientific, Inc.) using a Hewlett Packard HP5890A gas chromatograph equipped with a split injector and a flame ionization detector (FID). The following settings were used: injection port and detector temperatures = 250 °C, split head pressure = 17.5 psi, total helium flow rate = 65 mL/min, column throughput = 1.4 mL/min, inlet purge delay = 0.3 min. Samples (1 μL) were injected after suitable dilution in chloroform. The temperature gradient consisted of equilibration at 100 °C for 1 min, heating to 200 °C at a rate of 50 deg/min, and heating at a rate of 2 deg/min to a final temperature of 236 °C. Chromatograms and peak integration were recorded by use of either an HP3390A or an HP3394A Integrator. The (*R*,*R*/*S*,*S*) and (*R*,*S*/*S*,*R*) diastereomers eluted with retention times of 17.93 and 18.52 (±0.05) min, respectively. The relative amounts of substrate and product derivatives were quantitated by integration of the FID tracing. Ratios were obtained reproducibly within ±0.1 fraction percent, provided that the peak areas were within the linear range of the integrator (1700 to ~1.2 × 10^6 integration units; Powers, 1989). The ratios were calculated as the average of two or three separate runs. Control experiments showed that both the commercially supplied resolved mandelates and also the resolved MTPA acid chloride preparations were greater than 99% enantiomerically pure. The diastereomers of the derivatized mandelates elicited identical FID responses. Other controls verified that the diastereomeric compositions of the derivatives from the derivatization procedure accurately reflected the enantiomeric compositions of the initial mixtures of mandelate (Powers, 1989). Aliquots in which substrate turnover had proceeded to 5–8% were set aside for mass spectrometric analysis.

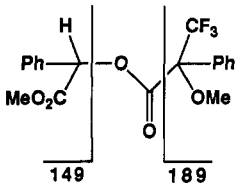
Table I: GC/Mass Spectrometry Data from the Hydrogen Tracer Experiments[a]

	% turnover (apparent)	M^{149}	M^{150}	final % H/(H + D)[b]
A. (*R*) to (*S*) Data (D_2O)				
(*R*)-mandelate controls				
no D_2O, no enzyme	0.36[c]	100	10.9	(100)[d]
in D_2O, no enzyme	0.31[c]	100	10.8	(100)[d]
remaining (*R*)-mandelate				
after 5.6% turnover	5.6	100	11.0	99.8
after 8.0% turnover	8.0	100	11.2	99.6
product (*S*)-mandelate				
after 5.6% turnover	5.6	8.3	100	1.7
after 8.0% turnover	8.0	6.3	100	1.8
B. (*S*) to (*R*) Data (D_2O)				
(*S*)-mandelate controls				
no D_2O, no enzyme	0.98[c]	100	10.9	(100)[d]
in D_2O, no enzyme	1.01[c]	100	10.7	(100)[d]
remaining (*S*)-mandelate				
after 6.1% turnover	6.1	100	14.4	96.5
after 8.2% turnover	8.2	100	16.2	94.9
product (*R*)-mandelate				
after 6.1% turnover	6.1	22.6	100	2.5
after 8.2% turnover	8.2	16.7	100	2.4
C. (*S*) to (*R*) Data (H_2O)				
(*S*)-mandelate controls				
in H_2O, no enzyme	0.18	1.9	100	98.1
remaining (*S*)-mandelate				
after 6.8% turnover	6.8	4.1	100	96.0[e]
after 8.9% turnover	8.9	6.1	100	94.2[e]
product (*R*)-mandelate				
after 6.8% turnover	6.8	100	4.8	2.0
after 8.9% turnover	8.9	100	3.0	0.9

[a] Error estimates: ±0.05% for percent turnover measurements, and ±0.1 for hydrogen isotope measurements. See also Table II. [b] Calculated as explained under Materials and Methods. [c] The average of both enzyme-free controls was assumed for the amount of nonenzymatically produced product mandelate derivative in subsequent calculations. [d] The natural abundance of the M + 1 ion from M^{149} was assumed to be 10.8%. The tabulated values for M^{150} have not been corrected for the component contributed by the M + 1 ion from M^{149}. [e] Not corrected yet for the protium present initially in the (*S*)-[α-^2H]-mandelate. See Table II.

Mass Spectrometric Determination of the Isotopic Content of the Mandelate α-Hydrogen. Mass spectrometric data were obtained at the Mass Spectrometry Facility of the University of California at Berkeley on a 70-eV electron impact Hewlett Packard 59970 Workstation GC/mass spectrometer (cation mode) and are shown in Table I. The in-line GC was of the same make as that used for the GC analysis above and was equipped with an identical DB-5 capillary column. The in-

Scheme II



jector port and the transfer line temperatures were 280 °C, the split head pressure was 8 psi, and there was no inlet purge delay. GC separations of the mandelate derivatives were performed by use of a temperature gradient of 4 deg/minute from an initial temperature of 200 °C to a final temperature of 236 °C. The steeper gradient (relative to that used in the GC analyses above) minimized the total run time while still providing base-line resolution of the diastereomers. Retention times for the (*R*,*R*/*S*,*S*) and (*R*,*S*/*S*,*R*) diastereomeric pairs were 7.16 and 7.40 min, respectively.

Data for the H:D isotope ratio calculations were obtained by use of selected ion monitoring ions of *m*/*e* values of 149 and 150. These ions corresponded to the most prominent fragment ions ($C_9H_9O_2$ and $C_9DH_8O_2$ for [α-^{1}H]- and [α-^{2}H]mandelate, respectively) derived from the MTPA methyl ester mandelate derivatives that still contained the mandelate α-hydrogen (Scheme II). Although these fragments amounted to only 10% of the base peak (*m*/*e* = 189; Scheme II), the mass spectrometer was sufficiently sensitive to quantitate the desired ions as long as the enzyme incubations had been carried out to at least 5% turnover. The GC column was loaded to capacity to maximize the signal of the minor, product-derived diastereomer while still maintaining base-line separation from the major, substrate-derived diastereomer. A detector photomultiplier setting of 2300 V and a dwell time of 50 ms per ion were used. Including dead time, and monitoring *m*/*e* = 149, 150, and 148 (as a control), the total scan rate was 4.7 scan cycles per second. For the derivatives of the [α-^{1}H]mandelates, the observed abundance of M^{150} (10.8%) relative to that of M^{149} (100%) was in acceptable agreement with that predicted (10.23%) from the natural abundances of ^{2}H, ^{13}C, and ^{17}O for a $C_9H_9O_2$ species. No significant peaks were observed in the ion chromatograms of neighboring ions M^{148} and M^{151}. These findings suggested that only the $C_9H_9O_2$ species (in the case of the [α-^{1}H]mandelate derivative) contributed significantly to the observed M^{149} and M^{150} intensities. Moreover, the fragmentation patterns of the derivatized mandelates were the same for all four diastereomers, and the secondary isotope effect due to deuterium at the mandelate α-hydrogen position was found to be 1.0 (data not shown). Thus, the intensities of M^{149} and M^{150} could be used to quantitate the relative proportions of protium and deuterium, respectively, in the mandelate α-hydrogen once the M^{150} ion had been corrected for the M + 1 contribution due to the [α-^{1}H]-containing species.

The ion chromatograms for M^{149} and M^{150} were integrated manually as follows. First, a constant, low level of background noise (5–6 intensity units per ion per scan) from the high photomultiplier voltage was subtracted from all scans. Second, only scans with intensities well above the threshold of detection were included for integration to avoid underestimation of weak intensity values. Third, the M^{149} and M^{150} ion chromatograms of derivatives containing a substantial proportion of deuterium in the α-hydrogen had to be staggered by two scans prior to integration in order to offset the faster migration of the [α-^{2}H]-containing species relative to that of the [α-^{1}H]-containing species.[3] To adjust the summation of M^{150} to reflect only the contribution from deuterium in the α-hydrogen, the portion due to the natural abundance M + 1 ion from the [α-^{1}H]-containing fragment was calculated (=10.8% × $\sum M^{149}$) and subtracted from M^{150}. The summations of M^{149} and M^{150} were then normalized such that the larger of the two was set equal to 100 arbitrary units. The resulting quantities, termed $\sum M^{149}$ and $\sum M^{150}$, were taken to reflect the relative populations of α-protium and α-deuterium in the derivatized mandelates. The corrected fraction percent of protium in the α-hydrogen from each mandelate–MTPA derivative was calculated as $\sum M^{149}/(\sum M^{149} + \sum M^{150})$.

Table II: Summary of Solvent Hydrogen Incorporation in Substrates and Products[a]

A. 5 mM (*R*)- and (*S*)-[α-^{1}H]Mandelate in D_2O

direction	% turnover[b]	% H in product[b]	% D in substrate
(*R*) to (*S*)	5.3	1.8	0.2
	7.7	1.9	0.4
(*S*) to (*R*)	5.1	3.0	3.5
	7.2	2.7	5.1

B. 5 mM (*S*)-[α-^{2}H]Mandelate in H_2O

direction	% turnover[b]	% D in product[b]	% H in substrate
(*S*) to (*R*)	6.6	2.0	2.1
	8.7	0.9	3.9

[a] Error estimates (estimated standard deviations): ±0.05% for percent turnover measurements. Sensitivity limits (±estimated standard deviations): 2 ± 1% for percent minor hydrogen isotope in the product, and 0.10 ± 0.05% for percent minor hydrogen isotope in the remaining substrate. [b] Corrected for nonenzymatic turnover.

Correcting for Minor Chiral Contaminants from the MTPA and Mandelate Starting Materials. Despite the high chiral purities of the reagent MTPA and mandelate enantiomers, GC analysis of the MTPA–mandelate methyl ester derivatives revealed measurable levels of the minor diastereomers. The reaction of reagent (*R*)-mandelate with (*S*)-MTPA-Cl revealed contamination of the (*R*)-mandelate–(*S*)-MTPA by 0.36 ± 0.02% of the (*S*,*S*)- and/or (*R*,*R*)-diastereomers, while the reaction of (*S*)-mandelate with (*R*)-MTPA-Cl yielded contamination of the (*S*,*R*) adduct by 0.98 ± 0.03% of the (*S*,*S*) and/or (*R*,*R*) diastereomers (lower values than these were obtained later with newer reagents).

Since the product mandelates from the enzyme incubations acquired virtually all of their α-hydrogen from the solvent, the chiral contaminants accounted for a substantial portion of the apparent α-protium population in the product mandelate–MTPA derivatives.[4] To compensate, the observed protium content of the product mandelate was corrected as follows. If the protium population in the (*R*) product mandelate was 14.5% (calculated as described in the preceding paragraph), if the apparent extent of turnover was 8.2% (determined by GC relative to the total mandelate pool), and if the (*S*) substrate had been contaminated originally with (*R*)-mandelate

[3] Alterations in rates of migration upon deuteriation have been reported previously [for example, see Pereira et al. (1973)].

[4] Whether the chiral contaminants were derived from the reagent mandelate or the MTPA-Cl stock was inconsequential since in either case the end result was the same. If the contaminant arose primarily from, for example, (*R*)-mandelate in the reagent (*S*)-mandelate stock, then for the (*S*) to (*R*) experiment (which involved derivatization with (*R*)-MTPA-Cl), the contaminant would contribute to the (*R*)-mandelate–(*R*)-MTPA peak as the protium-containing species. Alternatively, if the contaminant arose primarily from, for example, (*S*)-MTPA-Cl in the (*R*)-MTPA-Cl stock, then it would still contribute to the (*R*)-mandelate–(*R*)-MTPA peak as the protium-containing species (but as the (*S*,*S*) diastereomer) since most of the residual (*S*) substrate from the incubation retained its protium.

to the extent of 0.995% (the average of the two enzyme-free controls), then the amount of racemase-generated protium in the product was equal to (0.145 − (0.995/8.2)) × 100% = 2.4%. Note that since the correction (0.121) is comparable to the measured protium content (0.145), the error in assessing the transfer of the α-hydrogen from substrate to product is necessarily large. Since the true extent of *racemase-mediated* turnover was 8.2 − 0.995 = 7.2% relative to the total mandelate pool, the actual amount of racemase-generated protium in the product expressed as a percentage of the total enzymatically produced product was 2.4 × (8.2/7.2) = 2.7% (see Tables I and II). This correction was negligible for the deuterium incorporation measurements for the remaining substrate.

Hydrogen Tracer Experiments in H_2O. To verify the results of the experiments in D_2O, the tracer experiments were also carried out by use of (*S*)-[α-^{2}H]mandelate in H_2O. The synthesis of (*S*)-[α-^{2}H]mandelate relied both on the commercial availability of enantiomerically pure (*S*)-mandelate and on the ability of the H297N mutant of MR to catalyze solvent exchange with the α-hydrogen of (*S*)-mandelate without catalyzing racemization (Landro et al., 1991). Accordingly, (*S*)-[α-^{1}H]mandelate (760 mg) was dissolved in D_2O (50 mL), and the pD was adjusted to 7.1 with NaOD. Following lyophilization, the mandelate was dissolved again in D_2O, the pD was adjusted to 7.5, and the exchange reaction was initiated by the addition of a stock solution of the H297N mutant in D_2O (2.4 mg/mL, 100 μL). After 24 h at 25 °C, the pD was adjusted to 2 with DCl, and the (*S*)-[α-^{2}H]-mandelate was recovered by ether extraction. Exchangeable deuterium was removed by three dissolution/lyophilization steps in H_2O. This material was used in exchange experiments carried out in H_2O in essentially the same way as described earlier for the experiments in D_2O. GC analysis using the methods described above revealed that the (*S*)-mandelate contained 98.1% deuterium in the α-hydrogen. Contaminating (*R*)-mandelate or, indistinguishably, contaminating (*S*)-MTPA-Cl in the (*R*)-MTPA-Cl used for derivatization[4] were present together at a level of 0.18%. Since this contamination contained >95% deuterium in its α-hydrogen, it is most likely that the chiral contaminant occurred in the (*R*)-MTPA-Cl. The presence of this contaminant was taken into account in the mass spectroscopic analyses.

Overshoot Experiments. Overshoot experiments were monitored by the circular dichroic assay (Sharp et al., 1979) using a JASCO 500A spectropolarimeter equipped with a thermostated cell holder. A time constant of 1 s and a sensitivity of 26 mdeg full scale were employed. Reactions (2.5 mL) were carried out at 25 °C in a 1-cm quartz cell in 94 mM HEPES in D_2O (99.8 atom % D), pD = 7.5, containing 5 mM (*R*)- or (*S*)-[α-^{1}H]mandelate and 3 mM $MgCl_2$. A stock solution of enzyme was prepared by the 20-fold dilution of a more concentrated stock solution (12 000 units/mL; 1050 units/mg protein) into reaction buffer. Racemization was initiated by the addition of 20 μL of the dilute stock (final active site concentration = 120 nM, assuming one active site per 39 000 MW monomer). The progress of racemization was monitored at 267.8 nm and recorded using an IBM XT computer interfaced to the spectropolarimeter. Racemization was deemed complete when the ellipticity had remained null for 20–30 min. Controls in H_2O were run in both directions to demonstrate the lack of an overshoot in the absence of D_2O. The amount of enzyme used in these controls was one-fourth of the amount used for the reactions in D_2O to offset the 4-fold faster rate of the enzyme in H_2O. Additional controls demonstrated that the enzyme retained full activity for the duration of these incubations (Powers, 1989).

The measured maximum amplitude of the overshoot expressed as the fraction of the ellipticity prior to racemization, Θ_{min}, is related to the primary kinetic isotope effect, α, by eq 1

$$\Theta_{min} = \frac{\alpha}{2-\alpha}(\alpha-1)^{-2/(\alpha-2)} + \frac{2-2\alpha}{2-\alpha}(\alpha-1)^{-\alpha(\alpha-2)} \quad (1)$$

for $\alpha \neq 2$ (Cleland, 1977). For the special case where $\alpha = 2$, $\Theta_{min} = -0.135$. Note that Θ_{min} ranges from 0 to −0.45 for α ranging from 1 to 6. The value of α is obtained from the graph presented by Cleland (1977).

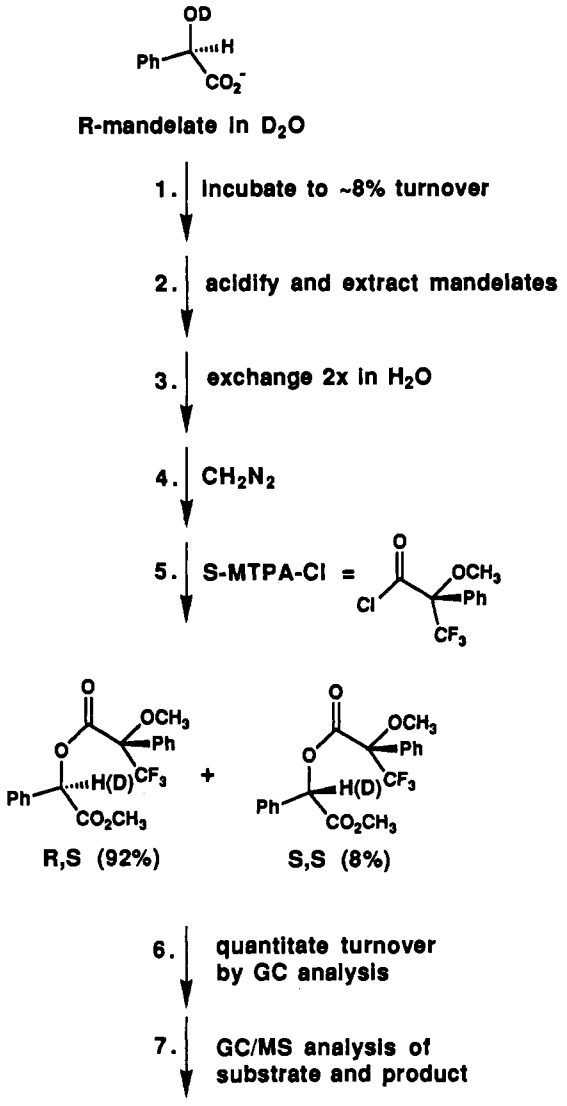
Results and Discussion

Rationale for the Tracer Experiment. To characterize the hydrogen-exchange patterns and, thus, to determine whether one or two bases directly mediate proton transfer in the reaction catalyzed by MR, a hydrogen tracer experiment was devised by use of α-protio-substrate and D_2O to establish both the source of the product α-hydrogen and the extent to which solvent hydrogen is exchanged into the remaining substrate. This particular isotope arrangement was chosen for initial experiments because of the commercial availability of highly enriched D_2O and enantiomerically pure (*R*)- and (*S*)-mandelates. Accordingly, (*R*)- and (*S*)-[α-^{1}H]mandelates were separately incubated with enzyme in buffered D_2O to small extents of turnover. Derivatization of the mandelate mixtures (after exchange of residual D_2O and esterification to mask the carboxyl groups) with a chiral derivatizing agent [(*R*)- or (*S*)-MTPA-Cl] rendered the mandelate enantiomers separable as diastereomers by GC so that the relative proportions of protium and deuterium in the α-hydrogens of the substrate and product mandelates could be analyzed independently by GC/mass spectrometry. This scheme is summarized in Scheme III. Although it was desirable to minimize the extent of turnover in the enzyme incubations to prevent individual mandelate molecules from experiencing multiple turnovers, the relatively low abundance of the ion fragment used for the hydrogen isotope measurements required that the reactions be carried out to at least 5% turnover. Accordingly, the aliquots removed at various times from the reactions were screened by GC to identify those that had been stopped after 5–8% turnover.

To complement the experiments involving [α-^{1}H]-containing substrate in D_2O, analogous experiments were also performed with (*S*)-[α-^{2}H]mandelate in H_2O. One advantage of these conditions was that the substrate constituted the sole source of deuterium in the experiment. The D_2O experiments described in the previous paragraph, on the other hand, were susceptible to contamination by atmospheric moisture. Contamination of the D_2O by H_2O to the extent of 1–2% might then account entirely for an observed level of internal return of comparable magnitude. The experiment in H_2O was made feasible by the availability of the H297N mutant of MR, which catalyzes the exchange of solvent hydrogen specifically into the α-hydrogen of (*S*)-mandelate without racemization (Landro et al., 1991). Mutant-catalyzed exchange in D_2O afforded enantiomerically pure (*S*)-[α-^{2}H]mandelate (99.8% (*S*); 98.1 atom % D).

Results of the Tracer Experiments. For the experiment in the (*R*) to (*S*) direction in D_2O (part A of Table II), the protium population in the product was quite low (<2%) after turnovers of both 5.3 and 7.7%. Since we estimate that the limits of detection of the minor isotope (protium) in the product mandelate is 2 ± 1%, no significant internal return (substrate α-protium transferred to the product) had occurred.

Scheme III



The protium content in the remaining (*R*) substrate rose only slightly (0.2 and 0.4%). Since we estimate that the limits of detection of the minor isotope (protium) in the substrate mandelate is 0.1 ± 0.05%, essentially all of the intermediate or enzyme-bound product reverting to free substrate was able to recapture the substrate-derived protion, and likewise, little or no product had reverted to substrate [an event that would produce (*R*)-[α-^{2}H]mandelate].

For the (*S*) to (*R*) direction in D_2O (part B of Table II), the product (*R*)-mandelate likewise contained a small quantity of protium in its α-hydrogen (3.0 and 2.7%) after turnovers of 5.1 and 7.2%, respectively. These values lie within the estimated limits of detection and, therefore, do not appear to provide evidence for internal return. However, the exchange into the remaining substrate was more substantial than that observed for the (*R*) to (*S*) direction. After 5.1% turnover, 3.5% of the total mandelate pool had been converted to α-deuterio-substrate, and a similar proportion (5.1%) was observed after 7.2% turnover.

Since the amount of protium detected in the product (*R*)-mandelate obtained from (*S*)-[α-^{1}H]mandelate was at the upper extreme of the estimated limit of detection and a potential source of error is contaminating H_2O in the D_2O solvent, a control experiment was performed with (*S*)-[α-^{2}H]mandelate in H_2O. This experiment (part C of Table II) gave results that were qualitatively similar to those with (*S*)-[α-^{1}H]mandelate in D_2O. Product (*R*)-mandelate contained only 1–2% deuterium, indicating negligible internal return, while solvent exchange into the remaining substrate amounted to ~2 and 4% of the remaining substrate after 6.6 and 8.7% actual turnover, respectively.

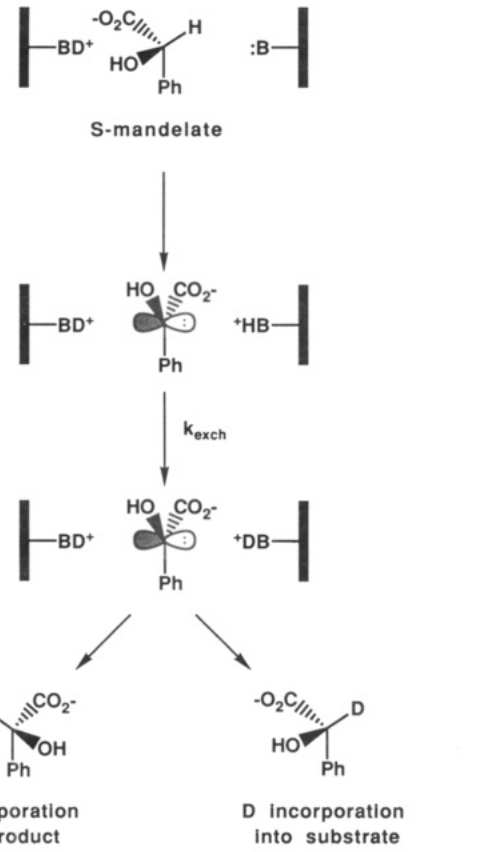
Interpretation. A one-base mechanism predicts a high degree of internal return, or, if most of the product α-hydrogen derives from solvent, predicts that the ratio of the rates of solvent exchange into the product and substrate is the same regardless of which enantiomer is used as substrate (Rose, 1966). Given an intermediate that equi-partitions between the forward and backward pathways (Scheme I), the latter case simplifies to requiring solvent hydrogen to appear in the product and the remaining substrate pools at comparable rates. From the results in Table II it is clear that neither condition is met with MR. Not only are the levels of internal return negligible for racemization in both directions, but also almost no solvent exchange occurs in the remaining (*R*) substrate in the (*R*) to (*S*) experiment. This finding is sufficient to rule out the one-base mechanism since although some exchange does occur in the remaining substrate of the (*S*) to (*R*) experiment, the one-base mechanism requires comparable exchange into both the product and the remaining substrate pools for the experiments in both directions. The results also rule out a mechanism involving "two bases in rapid communication," where two bases shuttle the substrate-derived protium atom from one side of the intermediate to the other with or without exchanging the proton with solvent (Sharp et al., 1977).

Instead, the results are fully consistent with a two-base mechanism. As expected, the product α-hydrogen is derived almost entirely from solvent, and negligible back-exchange occurs into the (*R*) substrate in the (*R*) to (*S*) experiment. Thus, the conjugate acid of the (*R*)-specific base is most likely monoprotic.

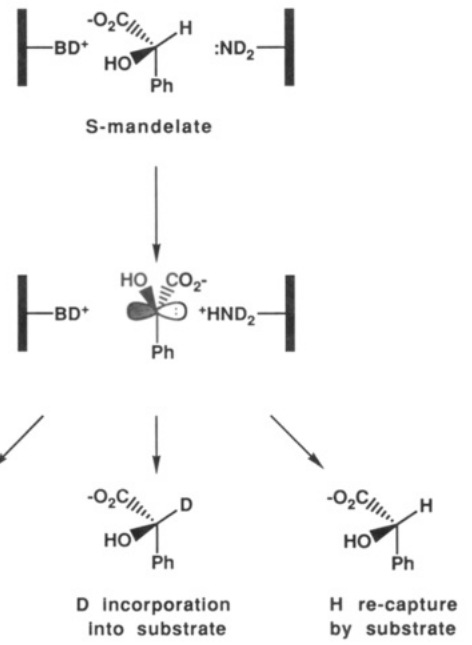
However, a significant amount of exchange does occur in the remaining (*S*) substrate in the (*S*) to (*R*) direction. Two alternative explanations could account for the latter observation (Schemes IV and V). Either the conjugate acid of the (*S*)-specific base exchanges the substrate-derived proton with solvent/active site protons rapidly on the time scale of the lifetime of the intermediate or it is polyprotic, e.g., an amino group. With the first alternative (Scheme IV; for illustrative purposes the reaction is shown to proceed via a carbanionic intermediate),[2] if the (*S*)-specific base were mobile at the stage of the intermediate, the base could exchange the substrate-derived proton with solvent or active site protons. Thus, the reversion of the intermediate to substrate would entail incorporation of solvent hydrogen, the degree to which would depend on the ratio of the exchange rate (k_{exch}) of the base relative to the rate of intermediate reversion to substrate.

In the second alternative (Scheme V; for illustrative purposes the reaction is shown to proceed via a carbanionic intermediate),[2] if the (*S*)-specific base were an amino group, rapid rotation of the ammonium group on the time scale of the intermediate would result in a 33% chance for the return of the substrate-derived proton to the reverting intermediate and a 67% chance for the acquisition of a solvent-derived proton neglecting kinetic isotope effects. The observation that about two out of every three (*S*)-substrate molecules (3.5/5.1 = 69%) that form from the collapse of the intermediate back to substrate exchange their α-protium atom with solvent deuterium

Scheme IV



Scheme V



(part B of Table II) is in surprisingly good agreement with this scenario. Since product dissociation does not appear to be rate-determining given the observed large substrate and solvent kinetic hydrogen isotope effects (C. W. Koo and G. L. Kenyon, unpublished observations), the absence of a kinetic discrimination in the ammonium group could be explained by the slow transfer of deuterium to the intermediate from the conjugate acid of the (*R*)-specific base. The values of 32 and 45% (=2.1/6.6 and 3.9/8.7, respectively; part C of Table II) found for (*S*)-[α-^{2}H]substrate in H_2O are also consistent with this explanation since under these conditions faster transfer of protium to the intermediate from the conjugate acid of the (*R*)-specific base could lead to an increase in the partitioning of the intermediate to product, thereby decreasing the flux of the intermediate back to substrate and diminishing the apparent degree of exchange into the remaining (*S*) substrate.

Since the conjugate acid of the (*R*)-specific base is deduced to be monoprotic and the conjugate acid of the (*S*)-specific acid is deduced to be polyprotic, the two catalytic bases are necessarily different residue types. Although this conclusion may appear to be counterintuitive, the active site environment could induce similar pK_a values and reactivities in different functional groups.

The substantial solvent exchange into the remaining (*S*) substrate is consistent with the existence of a discrete intermediate in the racemization reaction in accord with previous studies (Kenyon & Hegeman, 1979; Lin et al., 1988, 1991). Although reversion of bound product to substrate prior to dissociation would also produce the observed exchange with solvent, this is inconsistent with evidence that product dissociation is not rate-determining (C. W. Koo & G. L. Kenyon, unpublished observations). The question of whether an intermediate can be formed in the active site of MR is further considered in the third paper of three in this issue (Landro et al., 1991).

The Overshoot Experiment. In light of the exchange patterns deduced from the hydrogen tracer studies, the occurrence of an overshoot was sought for the racemization of (*R*)- or (*S*)-[α-^{1}H]mandelate in D_2O. The basis for this experiment, first applied in the studies of proline racemase (Cardinale & Abeles, 1968), was the following. Consider a two-base mechanism in which (1) two monoprotic bases (an acid/base pair) mediate racemization, (2) racemization proceeds with the incorporation of exactly one atom of solvent hydrogen into the product α-hydrogen regardless of the chirality of the substrate, (3) solvent hydrogen exchanges into the substrate pool only via the reversion of free product back to substrate, and (4) proton abstraction from substrate is a rate-determining step. If the racemization of an initially pure [α-^{1}H]mandelate enantiomer, the (*R*) enantiomer for example, is followed in D_2O until racemic [α-^{2}H]mandelate is obtained, then the course of racemization can be described by[5]

$$R_H \xrightarrow{k_1} S_D \underset{k_3}{\overset{k_2}{\rightleftharpoons}} R_D \qquad (2)$$

where the subscripts H and D indicate the α-hydrogen isotope and k_2 and k_3 are less than k_1. As shown explicitly by Cleland (1977), the time course of racemization can be predicted since the analysis of this scheme gives rise to a differential equation that can be solved for the total concentration of (*R*)- or (*S*)-mandelate as a function of time. Rather than describing an exponential decay toward an asymptote of racemic [α-^{2}H]mandelate, this scheme requires an eventual but temporary excess (overshoot) of the product enantiomer over the substrate enantiomer. The magnitude of this overshoot depends on how much slower k_2 and k_3 are than k_1 (i.e., on the magnitude of the primary deuterium kinetic isotope effect for each direction in D_2O). The overshoot can be rationalized as follows (Cardinale & Abeles, 1968; Cleland, 1977). Initially, the time course involves solely the conversion of (*R*)-[α-^{1}H]mandelate

[5] While the assumption, $k_2 = k_3$, holds approximately for MR, a better expression for each of the rate constants k_1, k_2, and k_3 would be of the form, $k_{cat}[S]/([S] + K_m)$, to take into account the dependence of the rate on substrate (or product) concentration. The initial substrate concentration was 5 mM, whereas the K_m values for the enantiomers of [α-^{1}H]- and [α-^{2}H]mandelate are approximately 0.25 mM (Whitman et al., 1985).

to (*S*)-[α-^{2}H]mandelate, but as the concentration of the product rises, the conversion of (*S*)-[α-^{2}H]mandelate to (*R*)-[α-^{2}H]mandelate becomes significant. When an (*R*):(*S*) ratio of 50:50 is first achieved, the α-hydrogen of the product (*S*) enantiomer contains only deuterium. However, some of the remaining substrate, having not yet been racemized, still contains protium, and on account of the primary kinetic deuterium isotope effect in D_2O, the (*R*) to (*S*) flux continues to outpace the flux in the reverse direction, resulting in an excess of (*S*)- relative to (*R*)-mandelate. Once the two fluxes become equal when the excess of (*S*)-mandelate balances the kinetic discrimination against (*S*)-[α-^{2}H]mandelate due to the isotope effect, the (*R*):(*S*) ratio gradually and asymptotically returns to 50:50.

In the case of MR, the hydrogen tracer results predict that the above scheme should not hold exactly. As noted above, the (*S*)-specific base exchanges solvent deuterium into the remaining substrate pool even when racemization proceeds to a low extent of turnover. This should have the effect of reducing the overshoot for the (*S*) to (*R*) experiment since much of the protium in the remaining (*S*)-mandelate will have been depleted by the time a 50:50 mixture of mandelates is reached for the first time. On the other hand, the overshoot in the (*R*) to (*S*) experiment should not be compromised whatsoever, since the (*R*)-specific base does not catalyze significant exchange into the remaining (*R*) substrate.

These are precisely the findings, as shown in Figure 1. An overshoot occurs for racemization in each direction, with the overshoot in the (*R*) to (*S*) direction being the more pronounced. Using the correlation between the magnitudes of the overshoot and the isotope effect (eq 1; k_2 and k_3 are presumed equal in this treatment), the magnitude of the overshoot in the (*R*) to (*S*) direction (θ_{min} = –0.092; Figure 1A) predicts a primary kinetic deuterium isotope effect of 1.7 for racemization in the (*R*) to (*S*) direction, while the magnitude from the (*S*) to (*R*) experiment (θ_{min} = –0.035; Figure 1C) predicts an isotope effect of 1.3 (a lower limit, as explained above) for the (*S*) to (*R*) direction. These diminished primary deuterium kinetic isotope effects relative to those measured in H_2O (Whitman et al., 1985) demonstrate that proton abstraction is not so rate-limiting in D_2O as it is in H_2O, implying that an intermediate is kinetically significant in the racemization reaction (C. W. Koo and G. L. Kenyon, unpublished observations).

These results agree with the solvent-exchange results and are, therefore, consistent with the two-base mechanism now inferred for MR. Overshoots have also been observed for the two-base enzymes proline racemase (Cardinale & Abeles, 1968) and hydroxyproline 2-epimerase (Finlay & Adams, 1970). We emphasize that an overshoot does not by itself prove a two-base mechanism. Given a primary kinetic isotope effect on hydrogen abstraction, an overshoot will also arise with a one-base mechanism: when a 50:50 substrate to product ratio is reached for the first time, processing of substrate will still outpace that of product due to the higher deuterium content of the latter. Indeed, α-amino-ϵ-caprolactam racemase, a one-base enzyme, exhibits a moderate overshoot even though racemization is accompanied by 17% internal return in the (*R*) to (*S*) direction (Ahmed et al., 1986). Nevertheless, the fact that the magnitudes of the overshoots in the opposite directions are different indicates that two bases, rather than one, mediate catalysis, since a single base would be expected to produce overshoots of identical magnitude.

The demonstration of overshoots for MR catalysis constitutes a marked departure from an earlier study that found no

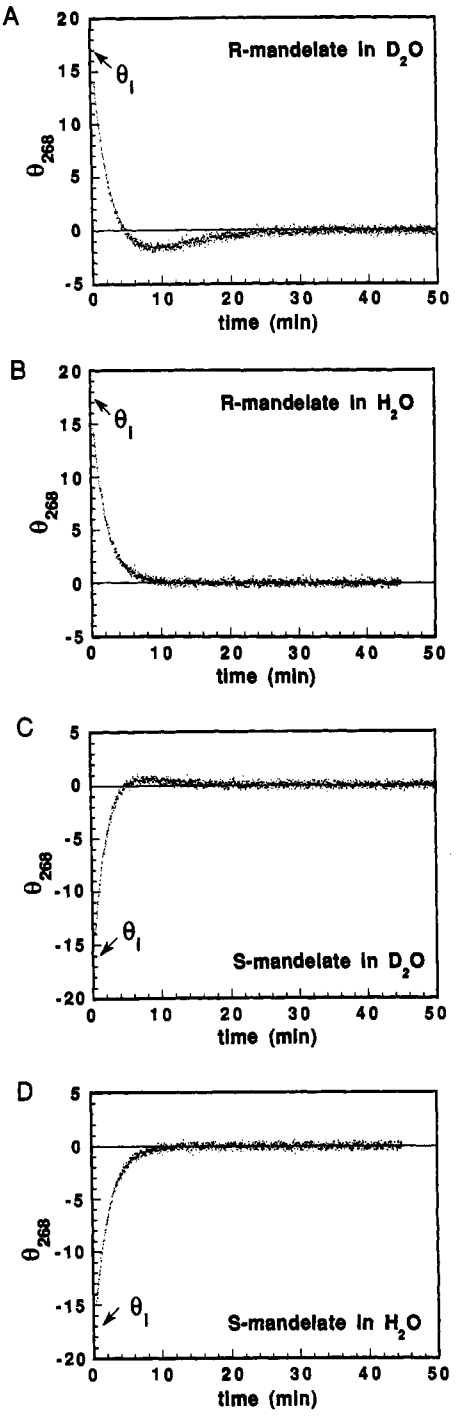


FIGURE 1: Results of the overshoot experiments. The MR-catalyzed racemization of (*R*)- or (*S*)-[α-^{1}H]mandelate in D_2O (or H_2O as a control) was followed using the circular dichroic assay described under Materials and Methods. (A) Racemization of (*R*)-[α-^{1}H]mandelate in D_2O. (B) Racemization of (*R*)-[α-^{1}H]mandelate in H_2O. (C) Racemization of (*S*)-[α-^{1}H]mandelate in D_2O. (D) Racemization of (*S*)-[α-^{1}H]mandelate in H_2O.

overshoot for racemization in either direction (Whitman et al., 1985). The likely causes of these earlier findings were the paucity of enzyme available at the time, the use of D_2O of only 95 atom % deuterium, and the intrinsically small sizes of the overshoots. The experiments in the earlier work were long in duration (10 h each), resulting in overshoots that were evidently too shallow to be detected.

Other Racemases and Epimerases. In addition to MR, several other racemases and epimerases involving intermediates have been characterized. Those with two-base mechanisms include proline racemase (Cardinale & Abeles, 1968; Rudnick

& Abeles, 1975; Albery & Knowles, 1986), hydroxyproline 2-epimerase (Finlay & Adams, 1970), diaminopimelic acid epimerase (Wiseman & Nichols, 1984), methylmalonyl-CoA epimerase (Leadley & Fuller, 1983; Fuller & Leadley, 1983), and ribulose-5-phosphate 3-epimerase (Davis et al., 1972). All exhibit negligible levels of internal return and all but one (diaminopimelic acid epimerase) show negligible solvent exchange into the remaining substrate. Asymmetry in the exchange of solvent hydrogen into the remaining substrate appears to be unique to MR, however. The class of one-base racemases and epimerases is limited so far to α-amino-ϵ-caprolactam racemase and several alanine racemases, all of which are pyridoxal phosphate dependent. Both α-amino-ϵ-caprolactam racemase (Ahmed et al., 1986) and *Pseudomonas striata* alanine racemase (Shen et al., 1983) display 10–20% internal return under certain conditions, and affinity labeling experiments with several alanine racemases have identified the lysine that tethers pyridoxal phosphate to the enzyme as the likely base (Faraci & Walsh, 1989, and references cited therein). Presumably, a single base is sufficient to mediate proton abstraction and redeposition in the one-base enzymes because of the stability of the PLP–aldimine intermediate, while two bases are required when the intermediate is not so stable [discussed most recently by Faraci and Walsh (1988)].

In spite of the many studies on the two-base enzymes to date, persuasive evidence regarding the identities of the catalytic bases has been presented only for proline racemase and hydroxyproline 2-epimerase. Two cysteines, one from each identical subunit in the dimer, are thought to constitute the two bases in proline racemase (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975; Albery & Knowles, 1986), while two cysteines from the same polypeptide chain are thought to be involved in monomeric hydroxyproline 2-epimerase (Finlay & Adams, 1970; Zervos & Adams, 1975). In contrast, it is doubtful that MR employs cysteine as a base since not only may the enzyme be purified in the absence of reducing agents (Tsou et al., 1989) but it also resists inactivation by the thiol labeling agents sodium tetrathionate, methyl methanethiosulfonate, and Ellman's reagent (Powers, 1989).

Conclusion. The results from the present study show that two bases mediate proton abstraction and redeposition in the reaction catalyzed by MR. The (*R*)-specific base does not catalyze significant solvent exchange into the α-hydrogen of the remaining (*R*) substrate and thus is probably monoprotic. The (*S*)-specific base mediates significant solvent exchange into the remaining (*S*) substrate and *may be* an amino group. The following papers (Neidhart et al., 1991; Landro et al., 1991) reveal the three-dimensional structure of the enzyme at atomic resolution and address directly the question of the possible identities of the chemical components in the catalytic mechanism.

ACKNOWLEDGMENTS

We thank Dr. E. Thomas Everhart for recommending the use of Mosher's reagent (MTPA-Cl); Sviatoslav Bozhenko and members of the Ortiz deMontellano lab (Yearn Choe, Julie Freutel, and Gia Depillis) for making their gas chromatographs available to us; Mr. Paul deFur and Sherry Ogden of the UC Berkeley Mass Spectrometry Facility for training us in the use of the GC/mass spectrometer; and Dr. Everhart and Professor Gregory A. Petsko for many helpful discussions. We also thank James Landro for synthesizing the sample of (*S*)-[α-^{2}H]mandelate.

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