Racemization of Vinylglycolate Catalyzed by Mandelate Racemase

Rongshi Li,[1a] Vincent M. Powers,[1a] John W. Kozarich*,[1b] and George L. Kenyon*,[1a]

Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143 and Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742

Received January 23, 1995[⊗]

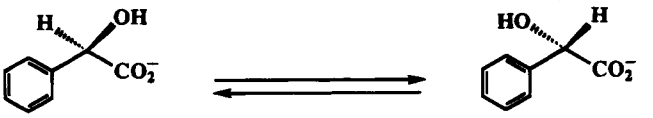
Vinylglycolate (2-hydroxy-3-butenoic acid, **2**) has been found to be an excellent substrate of mandelate racemase. The measured steady-state kinetic parameters for the enantiomers of **2** are comparable, with a maximal racemization rate that is 35% relative to mandelate. Racemization of **2** is subject to a primary deuterium kinetic isotope of about 4, indicating that abstraction of the α-proton is at least partially rate-limiting. Although α-hydroxybutyrate (**5**), the saturated analogue of **2**, is not a substrate, **5** competitively inhibits racemization of **2** with a K_i value comparable to the average K_m value for the latter. These results implicate the importance of β,γ-unsaturation in promoting facile racemization of the substrate α-proton. In addition, the enzyme catalyzes the isomerization of **2** to α-ketobutyrate (**4**), with a partition ratio for racemization/isomerization of about 1×10^4. These observations highlight the precision with which mandelate racemase can promote racemization to the virtual exclusion of a thermodynamically more favored process.

Introduction

Mandelate racemase (EC 5.1.2.2; MR) from *Pseudomonas putida* catalyzes the interconversion of the (*R*)- and (*S*)-enantiomers of mandelic acid (**1**; Scheme 1). The enzyme is Mg^{2+}-dependent, and the reaction proceeds *via* abstraction of the α-proton from either enantiomer of **1**, followed by stereorandom reprotonation of a transient enolic intermediate. Although the symmetrical kinetic parameters observed for the enantiomers of **1**[2] suggests corresponding structural symmetry in the active site, recent studies have shown otherwise. Kinetic and X-ray crystallographic studies on the native enzyme and selected mutants indicate the presence of two distinct acid/base residues in the active site: an imidazole group (His297) which effects abstraction/addition of the α-proton on the "(*R*)-face" of **1**, and an amino group (Lys166) which operates on the "(*S*)-face".[3-7] Catalysis may also be facilitated by coordination of hydroxyl and carboxylate oxygens in **1** with the active site Mg^{2+} ion and by protonation of a second carboxylate oxygen of **1** by the carboxyl group of Glu317 to promote substrate enolization.[6,7]

In earlier studies, it was shown that MR exhibits broad specificity for racemization of a variety of aromatic-substituted α-hydroxy acids, whereas other analogs, including the α-methyl analog of **1** (lactic acid), are inactive as substrates.[8,9] Although the basis for substrate inactivity was not established, it was thought that the α-phenyl group in **1** facilitates abstraction of the α-proton via electron delocalization of an incipient carbanionic intermediate,[8] now considered an *enolic* intermediate.[7] More recently, an acetylenic analogue of mandelate, propargylglycolate, was reported to be a moderately good substrate for racemization, with a turnover rate for racemization of about 10% relative to mandelate. Propargylglycolate was also found to be an irreversible inhibitor, with a partition ratio of racemization/inactivation of about 17 000.[10]

Scheme 1



(*R*)-1 (*S*)-1

In the present study, we have investigated vinylglycolate (2-hydroxy-3-butenoic acid, **2**) and its saturated analogue, α-hydroxybutyrate (**5**), as potential substrates of MR to probe the importance of β,γ-unsaturation in catalysis. We report that **2** is a good substrate for racemization, while **5**, though not a substrate, is a competitive inhibitor. Moreover, **2** undergoes slow but detectable enzyme-catalyzed isomerization to form α-ketobutyrate (**4**), a reaction which links MR to the isomerases.

Experimental Section

General. All chemical starting materials were obtained from Aldrich, except for 2-hydroxybutanoic acid (Sigma Chemical Co.). Diazomethane was prepared as an ethanol-free ethereal solution from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) according to directions supplied on the bottle.

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1995.

(1) (a) University of California, San Francisco. (b) University of Maryland, College Park. (c) Abbreviations: CD, circular dichroism; ee, enantiomeric excess (% major enantiomer minus % minor enantiomer); FID, flame ionization detector; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); KIE, kinetic isotope effect; LDH, lactate dehydrogenase; LSIMS, liquid secondary ion mass spectrometry; MR, mandelate racemase; MTPA, α-methoxy-α-(trifluoromethyl)-phenylacetic acid; *o*-PDA, *o*-phenylenediamine; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; VGA, vinylglycolate.

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(*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) were prepared from the respective free acids (>99% ee) according to the method of Dale et al.[11] α-Phenylglycidate was prepared as described by Fee et al.[12] Wheaton Reacti-vials (1 mL size) were from VWR. D-Lactate dehydrogenase from *Leuconostoc mesenteroides* and L-lactate dehydrogenase from porcine heart were obtained from Sigma Chemical Co.

NMR spectra were obtained at UCSF on a home-built 240 MHz wide bore NMR spectrometer equipped with a Nicolet 1180 computer and a 293-B pulse programmer. Negative LSIMS (liquid secondary ion mass spectrometry) spectra were obtained at the UCSF Mass Spectrometry Facility, A. L. Burlingame, Director. Gas chromatographic analyses were performed with a DB-5 column (30 m, 0.32 mm i.d., J & W Scientific, Inc.).

Enzyme Preparation. Mandelate racemase (MR) was purified as described earlier.[13] Purity was estimated to be greater than 95% based on SDS polyacrylamide gel electrophoresis with Commassie blue staining. Enzyme concentrations were determined by the method of Lowry.[14] The specific activity of the enzyme was found to be 1040 U/mg, as measured by circular dichroic assay using 5 mM (*R*)-**1** as substrate.[3,15]

Synthesis of Vinylglycolate (2). Vinylglycolate was synthesized by modification of a published procedure.[16] To a stirred solution of 20.0 g (160 mmol) of 2-acetoxy-3-butenenitrile was added dropwise 20 mL of concentrated HCl over the course of 30 min at room temperature. After the addition was complete, the solution was stirred for 2 h at 70−75 °C. The resulting dark brown mixture was allowed to cool to room temperature, and water was added in amount minimally sufficient to dissolve the ammonium salts completely. The solution was then extracted 10 times with 30 mL portions of ether, and the ethereal layers were combined, dried over $MgSO_4$, and concentrated under reduced pressure to give 16.1 g of crude acid. The zinc salt was prepared by adding the free acid to a solution of 6.50 g (80 mmol) of zinc oxide in 10 mL of water. The mixture was stirred at 90 °C for 1 h and then allowed to cool to room temperature, giving a semisolid mass. Absolute ethanol (12 mL) was then added, and the suspension was stirred for 2 h. The solid was isolated by filtration and washed with small portions of ethanol and ether. Three recrystallizations from water gave 10.1 g of fine, white crystals. The free acid was obtained by dissolving the zinc vinylglycolate in 30 mL of 2.5 M sulfuric acid and extracting 10 times with 25 mL portions of ether. The combined ethereal layers were dried over $MgSO_4$ and concentrated to give 6.2 g of the pure acid. Overall yield: 38%; ^{1}H NMR (D_2O) δ (ppm) 5.92 (m, 1H), 5.37 (d, J = 15.1 Hz, 1H), 5.26 (d, J = 9.6 Hz, 1H), 4.69 (d, J = 5.5, 1H).

Resolution of (*R*)- and (*S*)-Vinylglycolate. The enantiomers of **2** were resolved by cocrystallization with (*R*)-(+)- or (*S*)-(−)-α-methylbenzylamine as follows. To crystallize the (*S*)-enantiomer of **2**, a solution of 4.0 g (40 mmol) of racemic **2** in 50 mL of water was titrated to pH 7.0 with a 1 M solution of (*R*)-(+)-α-methylbenzylamine in ethanol. The solvent was removed under reduced pressure to yield 8.6 g of crude salt, which was then recrystallized from 40 mL of ethyl acetate/ether (50:50) to give 6.1 g of white, crystalline needles. The recovered salt mixture was recrystallized five times from ether/absolute ethanol (100:8), yielding 510 mg of the diastereomeric salt, mp 119.5−120 °C. Each crystallization required one day. After the fifth crystallization, the free acid was liberated by dissolving the diastereomeric salt (250 mg) in 1.5 mL of 2.5 M sulfuric acid, extracting with 7 × 2 mL of ether, drying the combined ethereal layers over $MgSO_4$, and removing the ether under reduced pressure, yielding 110 mg of (*S*)-**2**. The (*R*)-enantiomer of **2** was resolved similarly using (*S*)-α-methylbenzylamine.

Stereochemical Determinations. The stereochemical configurations of the (*R*)- and (*S*)-enantiomers of **2** were determined spectrophotometrically using D- and L-specific lactate dehydrogenases.[17] The enantiomer recognized by the L-specific dehydrogenase from *Leuconostoc mesenteroides* (*vide supra*) was assigned the (*S*)-configuration, and the other enantiomer, recognized by the D-specific dehydrogenase from porcine heart (*vide supra*), the (*R*)-configuration.

Purity of Enantiomers of 2. The enantiomeric purities of the resolved enantiomers of **2** were determined by gas chromatography after derivatization with (*R*)- or (*S*)-MTPA-Cl as follows. To a Reacti-vial (1 mL size) containing dry (*R*)- or (*S*)-**2** (5 mg, 50 μmol) was added by syringe a slight excess of ethereal diazomethane (~1 mL) such that the yellow color characteristic of diazomethane remained after nitrogen evolution had ceased. After 10 min, the remaining ethereal diazomethane was removed by nitrogen stream. To the resulting oil were added 250 μL of carbon tetrachloride, 250 μL of pyridine (dried over KOH), and 19 μL of MTPA-Cl (94 μmol, 1.9 equiv). (*R*)-MTPA-Cl was reacted with the (*S*)-enantiomer of **2**, and (*S*)-MTPA-Cl with the (*R*)-enantiomer of **2**, so that the less prevalent diastereomeric product in each instance would elute first during gas chromatography. Following addition of the appropriate enantiomer of MTPA-Cl, the vial was capped, shaken briefly, and kept in darkness at room temperature for approximately 18 h. The ethereal layer was then extracted four times with 4 mL portions of 0.5 M HCl, three times with saturated sodium carbonate, and once with water. After drying over $MgSO_4$, the ethereal solution was concentrated to dryness by nitrogen stream.

The diastereomeric derivatives of (*R*)- and (*S*)-**2** were resolved and quantified using the gas chromatography system above, with a column carrier flow rate of 1.4 mL helium/min, and a temperature gradient consisting of 100 °C for 1 min, 30 °C/min to 160 °C, and 2 °C/min to 220 °C final. Under these conditions, the diastereomers were base line resolved, with retention times of 16.1 and 16.6 min for the (*R*),(*R*)/(*S*),(*S*) and (*R*),(*S*)/(*S*),(*R*) diastereomeric pairs, respectively. Based on this method, the estimated purities of (*R*)- and (*S*)-**2** were as follows: (*R*)-**2**, 98.0 ± 0.2% ee; (*S*)-**2**, 98.8 ± 0.2% ee.

Synthesis of (*R*)- and (*S*)-α-[^{2}H]-2. An aqueous solution (50 mL) containing 5.0 g of racemic **2** (1 M), 20 mM sodium pyrophosphate, and 3 mM $MgCl_2$ in D_2O (99.8 atom % D) was incubated at 25 °C with 5.5 mg (6000 units) of mandelate racemase for 72 h. Acidification followed by ether extraction yielded 4.0 g of deuteriated **2** (80% yield). Both ^{1}H-NMR and negative probe liquid secondary ion mass spectrometry analyses indicated that the level of incorporation of deuterium in the α-proton was ~91%. The enantiomers of the α-[^{2}H]-product were isolated by cocrystallization with α-methylbenzylamine as above. On the basis of the gas chromatographic procedures described above, the enantiomeric purities were as follows: (*R*)-[^{2}H]-**2**, 98.4 ± 0.2% ee; (*S*)-[^{2}H]-**2**, 98.0 ± 0.2% ee.

Kinetic Studies. Steady-state kinetic parameters (k_{cat} and K_m) for racemization of **2** by MR were determined by modification of the circular dichroic assay of Sharp et al.[15] Assay solutions consisted of (*R*)- or (*S*)-**2** (0.5 to 8 mM), Tris-acetate[18] (94 mM, pH 7.5), $MgCl_2$ (3 mM), and an appropriate concentration of racemase. The assays were carried out at 25 °C in a 1 mm or 10 mm pathlength cell, depending on the concentration of **2**, and the change in dichroism at 216 nm was monitored as a function of time. The measured molar ellipticities ($[\theta]^{216}$) for (*R*)- and (*S*)-**2** were found to be −1100 and +1120 deg cm^2 dmol, respectively, as measured at 25 °C in 20

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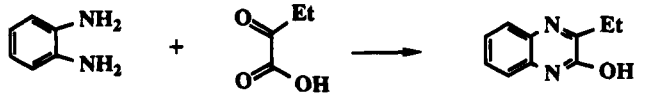
(18) TRIS buffer was employed in place of HEPES buffer because of the high UV absorbance of the latter. This substitution had no effect on the rate of racemization of **1**.

Scheme 2



o-PDA 4 λ_{max} = 334 nm

mM Tris-Acetate, pH 7.5. Racemization reactions were allowed to proceed to ≤10% conversion (substrate enantiomer to product enantiomer) over 5–20 min. The data were fitted to the Michaelis–Menten equation in double reciprocal form,[19] and k_{cat} and K_m were determined by least squares linear regression analyses.

Studies with 2-Hydroxybutanoic Acid (5). To assess potential substrate activity, a solution of racemic **5** (60 mM) was prepared in D_2O (99.8 atom % deuterium) containing 20 mM sodium pyrophosphate and 3 mM $MgCl_2$ in a total volume of 1 mL. After adjustment of the pD to 7.9 (pH meter reading = 7.5), the solution was transferred to a 5 mm diameter NMR tube, and an NMR spectrum was recorded. After 10 μL (120 units) of racemase stock solution was added, spectra were collected periodically to monitor any decrease in peak intensity for the α-proton (δ 3.95 ppm). No change in peak intensity was detected after incubation for 6 h, during which time enzyme activity fell by about 30%. When an identical amount of **2** was tested with the same amount of enzyme, exchange of the α-proton (δ 4.69 ppm) with solvent deuterium was complete within 10 min.

The inhibitory properties of **5** with respect to racemization of **2** were determined using the circular dichroic assay described above. The concentrations of (*R*)- and (*S*)-**2** were 1, 2, and 4 mM, and the concentrations of racemic **5** were 0, 1, 4, and 8 mM. Velocity data were fitted to the standard Michaelis–Menten equation for competitive inhibition, and K_i values with respect to racemization of (*R*)-**2** and (*S*)-**2** were determined by plotting the apparent K_m versus inhibitor concentration, where the slope = K_m (true)/K_i.[19]

Conversion of 2 to α-Ketobutyrate (4). The rate of conversion of **2** to **4** by mandelate racemase was determined using an assay in which **4** is treated with *o*-phenylenediamine (*o*-PDA) to produce a chromophoric derivative, 2-ethyl-4-hydroxyquinoxazoline (Scheme 2).[20] Enzyme incubations were carried out at 25 °C in solutions (1 mL) containing 93 mM HEPES, pH 7.5, 3 mM $MgCl_2$, 5 mM racemic **2**, and 120 units of MR. Aliquots (100 μL) were removed periodically and added to a 0.5 mL masked quartz cuvette (1 cm pathlength) containing 0.4 mL of 2.5 mM *o*-PDA in 80% acetic acid. The cuvette was then placed in the cell holder of a UV-vis-spectrophotometer and incubated at 50 °C for 10 min, and the absorbance at 334 nm was recorded. Enzyme incubations were run for 80 min to afford about 7% conversion of **2** to **4**. The concentrations of **4** were determined with reference to a calibration curve generated under identical conditions without enzyme. No α-ketobutyrate formed in the absence of enzyme.

Inactivation of MR with α-Phenylglycidate. To determine whether the racemase and isomerase activities of **2** are susceptible to inactivation by the active-site-directed irreversible inhibitor, α-phenylglycidate,[12] MR was incubated for 1 h at 25 °C in the presence of α-phenylglycidate, after which the enzyme was assayed for racemization and isomerization activity with respect to (*R*)- or (*S*)-**2** as above. The incubation mixtures consisted of 9 mM α-phenylglycidate and 0.26 mM racemase (~10 mg/mL) in 50 mM Tris-acetate, 10 mM $MgCl_2$, pH 8.0, in a total volume of 11 μL. A control without inhibitor was also run.

Results

The kinetics of racemization of (*R*)- and (*S*)-vinylglycolate (**2**) obey Michaelis–Menten kinetics (Figure 1). For

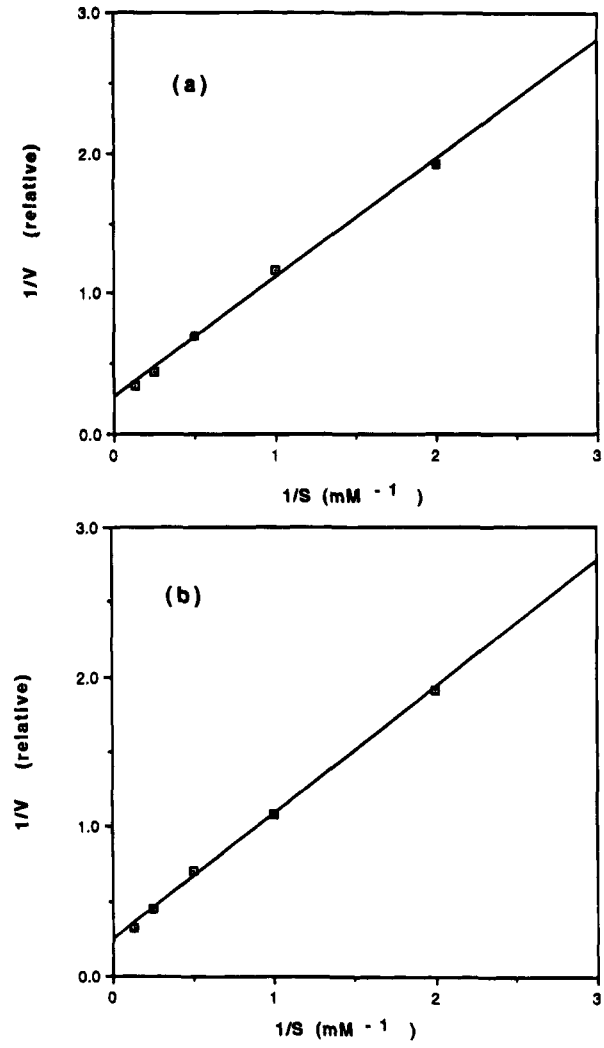


Figure 1. Lineweaver–Burk plots of the racemization of (a) (*R*)-vinylglycolate and (b) (*S*)-vinylglycolate by mandelate racemase in H_2O.

Table 1. Kinetic Constants for Racemization of (*R*)- and (*S*)-Vinylglycolate (VGA)

directions	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m	isotope effect k_{cat}	isotope effect k_{cat}/K_m
(*R*)- to (*S*)-α-VGA	240 ± 30	3.5 ± 0.4	70	3.9 ± 0.4	3.7 ± 0.4
(*S*)- to (*R*)-α-VGA	250 ± 20	3.5 ± 0.3	71	4.2 ± 0.4	3.7 ± 0.4
(*R*)- to (*S*)-α-[^{2}H]-VGA	62 ± 6	3.2 ± 0.3	19		
(*S*)- to (*R*)-α-[^{2}H]-VGA	60 ± 6	3.2 ± 0.4	19		

(*R*)-**2**, the measured k_{cat} and K_m were 240 ± 30 s^{-1} and 3.5 ± 0.4 mM, respectively (per 39 000 Da MR monomer). For (*S*)-**2**, the values were 250 ± 20 s^{-1} and 3.5 ± 0.3 mM (Table 1). These parameters compare favorably with those of (*R*)- and (*S*)-mandelate (**1**), for which k_{cat} and K_m are about 700 s^{-1} and 0.35 mM, respectively.[3] Since toluene and propylene have the same pK_a values (within 1 order of magnitude),[21] we can reasonably expect **1** and **2** also to have quite similar pK_a values. Apparently, the α-vinyl group in **2** provides roughly the same level of electron delocalization during catalysis as the α-phenyl group in **1**. However, the 10-fold difference in K_m values for **1** and **2** suggests the active site is better tailored for binding an α-phenyl group than a vinyl group.

Consistent with earlier isotope effect studies on **1** (deuterium KIE ~ 3.5[2]), a primary deuterium KIE of

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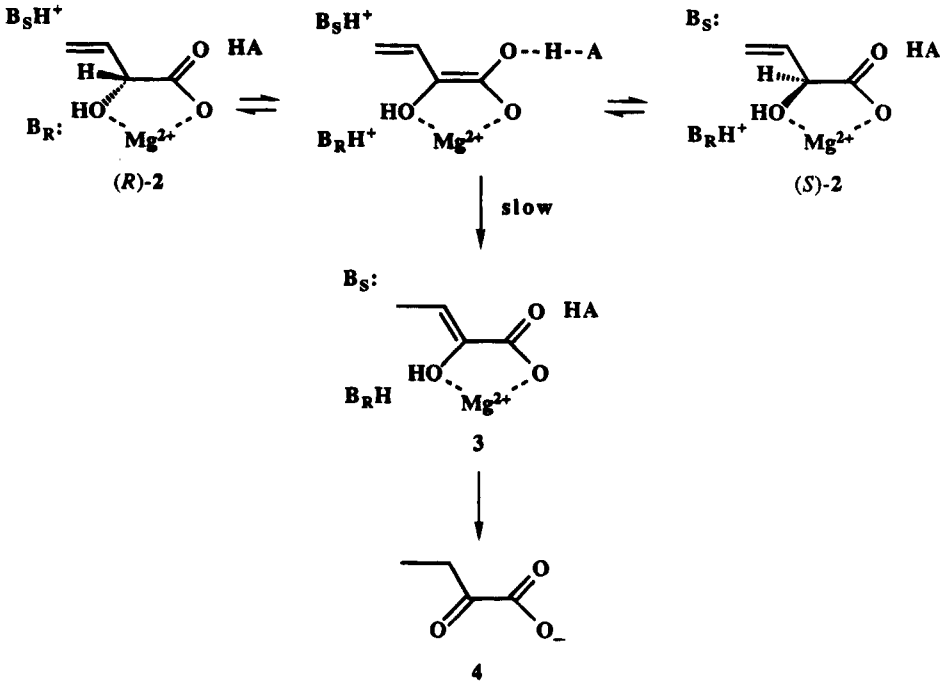
Scheme 3



about 4 was observed for racemization of (*R*)- and (*S*)-**2** (Table 1). This indicates that abstraction of the α-proton is only partially rate-limiting in the racemization of **2** (as with **1**) by MR, since full dependence on this step would be expected to give rise to an isotope effect on the order of 6–8.[22] The results are consistent with other chemical processes, such as protonation of the substrate carboxylate oxygen and/or reprotonation of the α-carbon in the enolic intermediate, as also being involved in the slowest step(s) in the reaction.

The substitution of the α-proton of **2** with deuterium had no effect on K_m, within the limits of experimental error. This result indicates that proton abstraction is much slower than the rates at which **2** is bound to and released from the active site and, therefore, that the K_m values for **2** represent simple dissociation constants (K_s).[23]

To examine the importance of β,γ-unsaturation to the rate of racemization of **2**, 2-hydroxybutanoic acid (**5**) was tested for substrate activity using a ^{1}H-NMR spectroscopic assay to monitor incorporation of solvent protons (from deuterium oxide) into the α-proton of **5**. Such incorporation necessarily occurs for each substrate molecule converted to its opposite enantiomer.[3] Despite an incubation in the presence of excess MR for a substantial period of time (6 h), no solvent incorporation (and hence, no racemization) into **5** was observed. From these results, an upper limit of about of 1×10^{-4} was established for the rate of racemization of **5** relative to **2**, assuming 70% enzyme activity during the course of the incubation,[24] and that a minimum of 10% incorporation could have been detected.

The lack of substrate activity of **5** is not attributable to low binding affinity in the active site. On the contrary, inhibition studies revealed that racemic **5** inhibits racemization of **2** competitively with an inhibition constant (K_i) of 3.6 ± 0.4 mM with respect to racemization of (*R*)-**2**, and a K_i of 3.8 ± 0.4 mM for racemization of (*S*)-**2**.[25] These values are substantially the same as the K_m values observed for **2**, suggesting that MR binds **2** and **5** with much the same affinity despite steric and stereoelectronic differences in their respective vinyl and ethyl substituents.

In light of earlier work reporting the MR-mediated conversion of *p*-(bromomethyl)mandelate to (*p*-methylbenzoyl)formate,[9] we expected that MR would also catalyze the isomerization of **2** to the thermodynamically more stable isomer, α-ketobutyrate (**4**). To this end, racemic **2** was incubated with an excess of MR, and aliquots of the product mixture were periodically removed for derivatization according to the reaction shown in Scheme 2. Formation of **4** was detected at a rate of about 1×10^{-4} relative to k_{cat} for racemization of **2** and only in the presence of enzyme.[26] In addition, both racemization of **2** and the formation of **4** were completely inhibited by pretreatment of enzyme with the active site affinity agent, α-phenylglycidate (2-phenyloxirane carboxylate), suggesting **4** arises as an occasional biproduct of racemization of **2** by MR.

Discussion

Although it was previously known that the phenyl group in mandelate (**1**) facilitates racemization by MR, the magnitude of this role was unclear. In the present case, we have shown that the vinyl analog **2** is an excellent substrate for racemization, whereas the saturated ethyl analog **5** is inactive by at least a factor of 10^4. Since the inhibition studies above showed that **5** binds to MR with substantially the same affinity as **2**, we conclude that the β,γ-unsaturation afforded by an α-phe-

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(24) Enzyme activity fell by 30% during the 6 h incubation period, irrespective of the presence or absence of **2**. We postulate the activity loss was due to exchange of solvent deuterium into exchangeable protons within or tightly associated with the MR polypeptide, causing undefined structural changes in the enzyme that affect catalytic activity.
(25) We assume that the enantiomers of **5** are equipotent inhibitors.
(26) The K_m for this process was not determined.

nyl, vinyl, or acetylenic group is essential for facile racemization by MR.

The limited impact on binding affinity observed on substitution of the phenyl group of **1** with a vinyl or ethyl group suggests that the other non-hydrogen substituents in these substrate compounds, i.e., the α-carboxyl and hydroxyl substituents, play greater roles in binding the substrate to the active site. This view is supported by recent high resolution crystallographic studies showing direct coordination of the required active site Mg^{2+} ion with both the α-hydroxyl oxygen and a carboxylate oxygen in two mandelate analogs.[7] The binding affinity of MR for substrate may be further enhanced by coordination of the second carboxylate oxygen of mandelate with the conjugate acid of Glu317.[7]

In addition to racemization, we have found that MR also catalyzes the isomerization of **2** to **4**, a thermodynamically favored process that proceeds at a rate that is about 10^4-fold slower than racemization. This process is consistent with the reaction scheme shown in Scheme 3. After **2** has been captured in the active site, the α-proton is abstracted by one of two enantiomer-specific bases (B_R or B_S) to produce a transient enolic intermediate. Formation of the enolic intermediate is thought to be facilitated by (i) coordination of the α-hydroxyl oxygen and a carboxylate oxygen with an active site divalent magnesium ion, and (ii) protonation of the second carboxylate oxygen by the conjugate acid of Glu317,[6a] designated HA in Scheme 3. Occasional protonation of the γ-carbon of the enolic intermediate, possibly by the conjugate acid of one of the enantiomer-specific bases (e.g., by B_RH^+ as shown in Scheme 3), affords 2-hydroxy-2-butenoate intermediate **3**. In turn, intermediate **3** tautomerizes to α-ketobutyrate **4**. The marked preference for racemization over isomerization of **2** provides a striking demonstration of the precision with which MR has evolved to catalyze racemization.

It is conceivable that **3** and **4** are formed after dissociation of the enolic intermediated from the active site, as was found in the case of a catalytically unproductive enediol intermediate that "escapes" from triose phosphate isomerase.[27] In the present case, however, it appears unlikely that the enolic intermediate formed upon abstraction of the α-proton of **2** could escape, since this intermediate would leave as a highly unstable dianionic carbanion or *aci*-carboxylate ($RCOO^{2-}$) species. Escape of the intermediate would also be disfavored for electrostatic reasons in light of the local positive charge generated by the divalent magnesium ion and the two protonated cationic bases B_R and B_S. Accordingly, we believe **3** forms while still bound to the enzyme active site, after which **3** may escape from the enzyme for tautomerization to **4**.

(27) Richard, J. P. *J. Am. Chem. Soc.* **1984**, *106*, 4926. Pompliano, D. L.; Peyman, A.; Knowles, J. R. *Biochemistry* **1990**, *29*, 3186.

Our observations with vinylglycolate bear comparison with the reactivity of MR toward several other mandelate analogs. Lin et al.[9] showed that MR converts *p*-(bromomethyl)mandelate to (*p*-methylbenzoyl)formate in a reaction involving a 1,6-elimination of hydrogen bromide followed by tautomerization of a *p*-xylylene intermediate. The rate of this process was estimated to be less than 1% of that for racemization, again showing the preference of MR for catalyzing racemization over other available isomerization reactions. Somewhat surprisingly, no inactivation of enzyme was observed despite generation of a reactive xylylene intermediate in the active site. This result can be understood if both catalytic bases in the active site are protonated in the presence of the intermediate (and hence are inactive as nucleophiles) and no other active site nucleophiles are nearby.

A different result was found with the α-acetylenic analog propargylglycolate, which is both a substrate and an irreversible inhibitor of MR.[10] Inactivation has been proposed to proceed through formation of an allenic enol intermediate which tautomerizes to 2-keto-3-butenoate, a strong Michael acceptor. Formation of the allenic enol may be promoted by the conjugate acid of either active site base, leaving that base free to react with the ensuing 2-keto-3-butenoate product. In contrast, enol intermediate **3** formed during isomerization of **2** is apparently not sufficiently reactive to bind covalently in the active site.

In contrast to the compounds discussed above, MR is rapidly inactivated by (*R*)-α-phenylglycidate, an epoxide analog of mandelate. In this case, the catalytic acid/base residues (His297 and Lys166) may work together to promote adduct formation, by protonation of the oxirane oxygen by the conjugate acid of His297 and concomitant nucleophilic attack at the oxirane β-carbon by the ε-amino group of Lys166. Interestingly, the (*S*)-α-phenylglycidate is inactive as an inhibitor for reasons that are not presently understood.

In conclusion, the present study has demonstrated the vital role of β,γ-unsaturation in promoting racemization by MR. The results above together with further studies will be useful in further defining the mechanisms of MR and related enzymes and in better understanding the means by which enzymes are able to abstract protons from carbon.

Acknowledgment. We thank Professor John C. Craig and Dr. E. Thomas Everhart for many helpful discussions. This work was supported by grants from the U. S. National Institutes of Health (GM40570 to G.L.K. and J.W.K., AR17323 to G.L.K., and GM 35066 to J.W.K.) and by a predoctoral fellowship from the American Foundation for Pharmaceutical Education (to V.M.P.).

JO950132O