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Redefining the Minimal Substrate Tolerance of Mandelate Racemase. Racemization of Trifluorolactate

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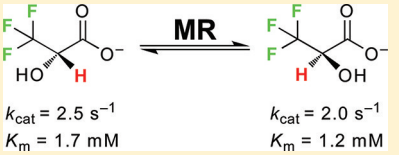
ABSTRACT: Mandelate racemase (EC 5.1.2.2) from *Pseudomonas putida* catalyzes the interconversion of the enantiomers of mandelic acid and a variety of aryl- and heteroaryl-substituted mandelate derivatives, suggesting that β,γ-unsaturation is a requisite feature of substrates for the enzyme. We show that β,γ-unsaturation is not an absolute requirement for catalysis and that mandelate racemase can bind and catalyze the racemization of (*S*)-trifluorolactate (k_{cat} = 2.5 ± 0.3 s^{-1}, K_m = 1.74 ± 0.08 mM) and (*R*)-trifluorolactate (k_{cat} = 2.0 ± 0.2 s^{-1}, K_m = 1.2 ± 0.2 mM). The enzyme was shown to catalyze hydrogen−deuterium exchange at the α-postion of trifluorolactate using ^{1}H NMR spectrocsopy. β-Elimination of fluoride was not detected using ^{19}F NMR spectroscopy. Although mandelate racemase bound trifluorolactate with an affinity similar to that exhibited for mandelate, the turnover numbers (k_{cat}) were markedly reduced by ∼318-fold, resulting in catalytic efficiencies (k_{cat}/K_m) that were ∼400-fold lower than those observed for mandelate. These observations suggested that chemical steps on the enzyme were likely rate-determining, which was confirmed by demonstrating that the rates of mandelate racemase-catalyzed racemization of (*S*)-trifluorolactate were not dependent upon the solvent microviscosity. Circular dichroism spectroscopy was used to measure the rates of nonenzymatic racemization of (*S*)-trifluorolactate at elevated temperatures. The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the nonenzymatic racemization reaction were determined to be 28.0 (±0.7) kcal/mol and −15.7 (±1.7) cal K^{-1} mol^{-1}, respectively, corresponding to a free energy of activation equal to 33 (±4) kcal/mol at 25 °C. Hence, mandelate racemase stabilizes the altered trifluorolactate in the transition state (ΔG_{tx}) by at least 20 kcal/mol.



Mandelate racemase (EC 5.1.2.2) is a member of the enolase superfamily of enzymes and catalyzes the Mg^{2+}-dependent 1,1-proton transfer which interconverts the enantiomers of mandelate (Scheme 1).[1] The ability of MR to catalyze rapid carbon−hydrogen bond cleavage from a carbon acid with a relatively high pK_a[2−4] makes it a useful paradigm for understanding enzyme-catalyzed proton abstraction from carbon acids.[1,4−7] Isotope exchange experiments, site-directed mutagenesis studies, and X-ray crystal structures of MR complexed with substrate and substrate analogues[8−12] indicate that catalysis proceeds via a two-base mechanism, with His 297 and Lys 166 abstracting the α-proton from (*R*)-mandelate and (*S*)-mandelate, respectively.[8,9,13] In addition, these experiments have revealed that Glu 317 acts as a general acid catalyst,[10] Lys 164 interacts with the carboxyl function of mandelate,[11] and Asn 197 interacts with the α-hydroxyl of mandelate to facilitate stabilization of the altered substrate in the transition state.[14]

MR can utilize a variety of aryl- and heteroaryl-substituted mandelate derivatives as substrates.[15−20] To date, the simplest substrate identified is vinylglycolate.[21] The ability of MR to racemize this substrate and not ethylglycolate[21] and lactate[19] has suggested that β,γ-unsaturation is required for racemization. Indeed, crystal structures with bound atrolactate reveal that this substrate analogue assumes a conformation within the active site such that proton abstraction is stereoelectronically favored (i.e., the plane of the phenyl ring is approximately perpendicular to the C_α−H bond, Figure 1). A comprehensive review of the substrate tolerance of MR conducted by Felfer et al.[15,22] led these authors to conclude that substrates of mandelate racemase must possess β,γ-unsaturation as a minimal requirement for activity. Herein, we show that the requirement for β,γ-unsaturation is not absolute and that stabilization of the negative charge of the enolic intermediate through inductive effects is also sufficient to promote racemization of a substrate. We show that MR accepts trifluorolactate (TFL) as a substrate and that the active site of the enzyme exhibits enhanced interaction with the trifluoromethyl group on the substrate relative to a methyl group.

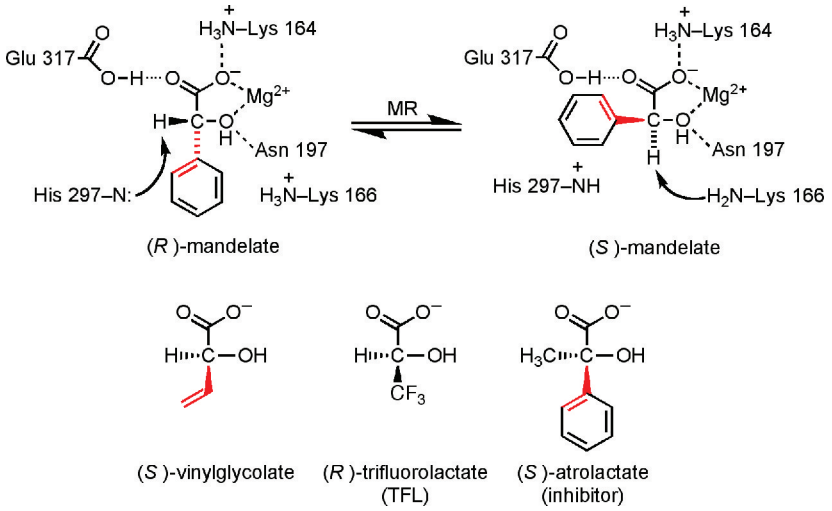
METHODS AND MATERIALS

(*R*)- and (*S*)-mandelic acid, (*S*)-trifluorolactic acid, and all other reagents, unless mentioned otherwise, were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). (*R*)-TFL was purchased from SynQuest (Alachua, FL). Circular dichroism (CD)-based assays and spectral measurements were conducted using a JASCO J-810 spectropolarimeter. NMR analyses were conducted at the Nuclear Magnetic Resonance Research Resource (NMR-3) using either a Bruker/Tecmag AC-250 spectrometer, Bruker AV-300 spectrometer, or Bruker AV-500 spectrometer.

Enzyme Purification. Recombinant MR from *Pseudomonas putida* was overexpressed in and purified from *Escherichia coli* BL21(DE3) cells transformed with a pET-52b(+) plasmid

Received: August 1, 2011
Revised: September 2, 2011
Published: September 6, 2011

Scheme 1. Reaction Catalyzed by Mandelate Racemase (MR) and Structures of Several Ligands[a]



[a] β,γ-Unsaturation is highlighted in red.

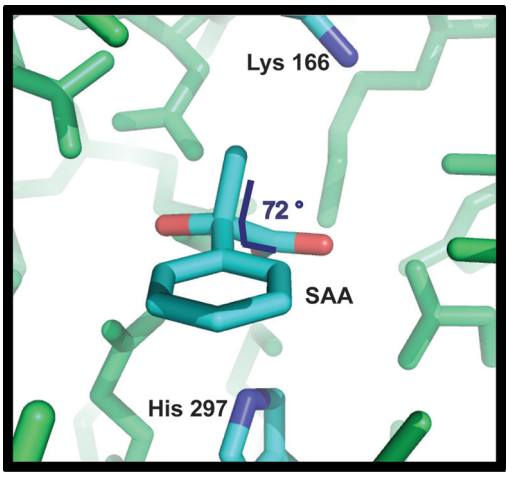


Figure 1. X-ray crystal structure of the active site of wild-type MR with bound (*S*)-atrolactate (SAA). The phenyl ring is approximately orthogonal to the C_α–CH_3 bond (PDB code 1MDR[9]). The active site (*R*)-specific and (*S*)-specific general acid/base catalysts His 297 and Lys 166 are shown, respectively. Oxygen, nitrogen, and carbon atoms are red, dark blue, and light blue, respectively. The α-carbon backbone is green. The dihedral angle for atoms between the *ortho* carbon of the phenyl ring and the CH_3 group is 72° as shown. The corresponding dihedral angles in the structures of the MR variants D270N (PDB code 1MRA[12]) and E317Q (PDB code 1DTN[10]) are 76° and 68°, respectively.

(Novagen, Madison, WI) containing the MR open reading frame as described previously.[23] This construct encodes the MR gene product as a fusion protein with an N-terminal StrepII-tag ($MASWSHPQFEKGALEVLFQGPGYHM_1$...MR, where M_1 denotes the initial Met of wild-type MR). The purity of the enzyme was assessed using SDS-PAGE (12% acrylamide) with staining by Coomassie blue R-250.[24]

Enzyme Assays. MR activity was assayed using a CD-based assay similar to that described by Sharp et al.[25] All assays were conducted at 25 °C in Na^+-HEPES buffer (0.1 M, pH 7.5) containing $MgCl_2$ (3.3 mM) (i.e., assay buffer) and bovine serum albumin (BSA, ≤ 0.01%). Initial velocities were determined by following the change in ellipticity at 262 or 228 nm when mandelate or TFL was the substrate, respectively, using a quartz cuvette with a 0.2, 0.5, or 1 cm light path with (*R*)-TFL, (*S*)-TFL, or mandelate as the substrate, respectively. The concentrations of mandelate (0.25–10.00 mM) and TFL (0.50–10.00 mM) were as indicated. Substrate solutions were incubated at 25 °C prior to initiation of the reaction by addition of freshly thawed enzyme solution to yield a final concentration of 0.15 or 33 μg/mL when mandelate or TFL was the substrate, respectively. The molar ellipticity for TFL at 228 nm was $[\theta]_{228}$ = 4404 deg mol^{-1} cm^2 (− for (*S*)-TFL and + for (*R*)-TFL).

Viscosity Effects. The dependence of MR-catalyzed racemization of (*S*)-TFL on solution viscosity was measured as described previously[20] using sucrose as the viscosogen. Briefly, stock solutions of varying concentrations of sucrose were prepared in assay buffer at twice the desired final concentration and subsequently diluted 1:1 with assay buffer. Reaction mixtures were prepared in rectangular quartz cuvettes with a 0.5 cm light path. Typically, 400 μL of (*S*)-TFL (0.5–20.0 mM) in Na^+-HEPES buffer (0.1 M, pH 7.5) containing $MgCl_2$ (3.3 mM) was mixed with 500 μL of the viscosogen-containing stock solution (prepared at twice the desired final concentration). The reaction was initiated by addition of 100 μL of wild-type MR (330 μg/mL) in HEPES buffer (0.1 M, pH 7.5) containing $MgCl_2$ (3.3 mM) and BSA (0.1%).

Data Analysis and Protein Concentrations. The values of V_{max} and K_m were determined from plots of the initial velocity (v_i) versus substrate concentration ([S]) by fitting the data to eq 1 using nonlinear regression analysis and the program *KaleidaGraph* v. 4.02 from Synergy Software (Reading, PA). All kinetic parameters were determined in triplicate, and average values are reported. The reported errors are standard deviations. Protein concentrations were determined using either the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada) with BSA standards or by measuring the absorbance at 280 nm using an extinction coefficient of 53 400 M^{-1} cm^{-1} that was calculated using the ProtParam tool available on the ExPASy server.[26] The values of k_{cat} were obtained by dividing V_{max} values by the total enzyme concentration ($[E]_t$) using M_r = 41 264.

$$v_i = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

dx.doi.org/10.1021/bi201188j | *Biochemistry* 2011, 50, 8846–8852

Product Analysis. To demonstrate that MR catalyzes the racemization of TFL and that the loss of ellipticity does not result from the β-elimination of fluoride, a product analysis was conducted. Two reaction mixtures (1.0 mL each) were prepared: a control containing 50 mM (*S*)-TFL in assay buffer and an MR reaction mixture containing 50 mM (*S*)-TFL and MR (59 μg/mL) in assay buffer. Each reaction mixture was incubated for 16 h at 25 °C. After the 16 h, the MR reaction mixture was passed through a Microcon Ultracel YM-10 centrifugal filter (10 kDa MWCO; Millipore, Billerica, MA) to remove the protein, yielding the MR reaction solution. D_2O, containing trifluoroacetic acid (TFA; 40 mM), was then added to the MR reaction solution and control solution, as an internal standard, to bring the final concentrations of D_2O to 25% (v/v) and TFA to 10 mM. The MR reaction and control solutions were subsequently analyzed using ^{19}F NMR spectroscopy (282.40 MHz).

Enzymatic H−D Exchange. The MR-catalyzed exchange of the α-proton of (*S*)-TFL with deuterium was conducted in buffered D_2O. The reaction was initiated by addition of MR (final concentration = 44 μg/mL) to 9 mL of (*S*)-TFL (35 mM) in 0.1 M tris(hydroxymethyl)aminomethane−DCl buffer (0.1 M, pD 7.5) containing 3.3 mM $MgCl_2$. The reaction was incubated at 25 °C in a water bath, and samples (1 mL) were removed at 0, 30, 60, 90, 120, 150, 180, and 210 min. The reaction was stopped by the addition of 20 μL of 1 M DCl to adjust the pD to 6.0, and the TFL was analyzed using ^1H NMR spectroscopy. The extent of deuterium incorporation into the α-position of TFL was calculated by comparing the loss of the integrated signal intensity of the TFL α-proton resonance with an electronic reference signal (corresponding to 8.58 mM) generated using the ERETIC method.[27,28] The observed first-order rate constant for deuterium incorporation was determined from linear plots of ln(α-proton integral) against time.

Nonenzymatic Racemization. The temperature dependence of the first-order rate constants for racemization of (*S*)-TFL (0.13 M) in potassium phosphate buffer (5 mM, pH 7.5) was determined by conducting reactions in sealed quartz tubes as described previously[29] at temperatures of 120, 130, 140, 150, 160, and 170 °C. Each tube contained 500 μL of (*S*)-TFL solution and was incubated at the appropriate temperature in a Thermolyne 47900 furnace. Tubes were removed at various time points, cooled to 4 °C, and stored at this temperature until analyzed using CD spectroscopy. The ellipticity was measured at 228 nm using a quartz cuvette with a 0.2 cm light path. The first-order rate constants for racemization (k_{rac}) were calculated from plots of ln θ_t vs t using eq 2, where $[S]_{int}$ is the initial concentration of (*S*)-TFL, $[S]_t$ is the concentration of (*S*)-TFL at time t, θ_{int} is the initial ellipticity of the (*S*)-TFL solution, and θ_t is the ellipticity of the (*S*)-TFL solution at time t.[30] The rate constant for enantiomerization (k_{enant}) was then calculated from k_{rac} as shown in eq 2.[30,31]

$$\ln\left|\frac{[S]_{int}}{[S]_{int}-2[S]_t}\right| = \ln\left|\frac{\theta_{int}}{\theta_t}\right| = k_{rac}t = 2k_{enant}t \quad (2)$$

RESULTS AND DISCUSSION

TFL as a Substrate. All substrates of MR reported to date have β,γ-unsaturation leading to the notion that delocalization of the negative charge generated upon general base-catalyzed deprotonation of the α-carbon is required for catalysis.[15−22] However, we anticipated that a suitable electron-withdrawing group, such as the trifluoromethyl group,[32] adjacent to the α-carbon could also promote catalysis. For example, the replacement of the methyl group of acetophenone by the trifluoromethyl group favors hydride transfer such that reduction of 2,2,2-trifluoroacetophenone is catalyzed by alcohol dehydrogenase from *Thermus thermophilus* HB27 while acetophenone is not a substrate.[33] Hence, we investigated the possibility that TFL could serve as a substrate for MR.

Indeed, MR catalyzes the racemization of (*R*)- and (*S*)-TFL as shown in Figure 2. Although we expected that the inductive

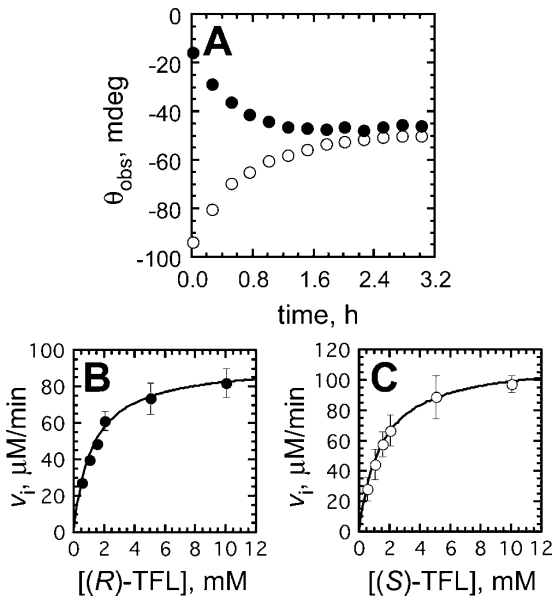


Figure 2. Racemization of (*R*)-TFL (●) and (*S*)-TFL (○) catalyzed by MR. (A) The change in ellipticity was followed over time starting with either (*R*)-TFL (10 mM) or (*S*)-TFL (10 mM) as the substrate. In addition to TFL, the reaction mixture contained MR (1.8 μg/mL) and BSA (80 μg/mL) in assay buffer. The sets of data do not converge at an ellipticity value of zero because the total ellipticity at 228 nm is offset by the presence of MR and BSA. In addition, the absolute ellipticity values corresponding to (*R*)- and (*S*)-TFL do not become equal at the 3 h time point due to the loss of enzyme activity. Upon addition of fresh MR, the absolute ellipticity values become equal. Representative Michaelis−Menten plots for the racemization of (*R*)-TFL and (*S*)-TFL are shown in (B) and (C), respectively. The experimental conditions are as given in the Materials and Methods.

stabilization afforded by the trifluoromethyl group would favor enzymatic proton abstraction, the fact that MR exhibits very low affinity for lactate ($K_i \approx 30$ mM)[19] suggested that it might also exhibit low affinity for TFL. Surprisingly, this was not the case. Indeed, the K_m values for (*R*)- and (*S*)-TFL are ~1−2 mM, similar to those for mandelate (Table 1). Previously, we showed

Table 1. Kinetic Parameters for the Racemization of Mandelate and Trifluorolactate by Mandelate Racemase

substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
(*R*)-mandelate	1.2 ± 0.2	792 ± 19	6.5 (±0.8) × 10^5
(*S*)-mandelate	1.0 ± 0.1	637 ± 31	6.2 (±0.8) × 10^5
(*R*)-trifluorolactate	1.2 ± 0.2	2.0 ± 0.2	1.6 (±0.3) × 10^3
(*S*)-trifluorolactate	1.74 ± 0.08	2.5 ± 0.3	1.4 (±0.2) × 10^3

that the dependence of binding free energies for glycolate analogues on π (π being the hydrophobicity substituent constant

dx.doi.org/10.1021/bi201188j | *Biochemistry* 2011, 50, 8846−8852

based on partitioning coefficients between octanol and water[34]) follows the equation $\log(K_m \text{ or } K_i) = (-0.72\ (\pm 0.08) \times \pi) - 1.4\ (\pm 0.1)$.[19] The steric volume of a CF_3 group is much larger than that of a methyl group, approximating that of an isopropyl group.[32,35] The value of π for the CF_3 group is 0.88,[34] indicating that the value of K_m for TFL should be ~10 mM (i.e., 10 ± 6 mM). Hence, MR appears to bind the CF_3 group of TFL with an affinity that exceeds that predicted from hydrophobic effects alone by about 5−10-fold. These calculations suggest that additional binding interactions may be involved in recognition of the CF_3 group, such as weak H-bond interactions[32] with the conjugate acid of one of the active site general base catalysts or orthogonal dipolar interactions with polarizable bonds on the protein.[35,36] Replacement of the methyl group of lactic acid by the trifluoromethyl group reduces the pK_a value of the carboxylic acid group from 3.86 in lactic acid[37] to 2.75 in *rac*-TFL.[38] Although this reduction in pK_a of the carboxylic acid likely has little effect on binding, the accompanying reduction in the pK_a value of the α-hydroxyl group[35] could result in stronger interactions with the magnesium ion within the active site. It is surprising that MR, as a member of the enolase superfamily, exhibits enhanced affinity for TFL relative to lactate. Whereas yeast and muscle enolase have been shown to bind the 2-phosphoglycolate analogue $\mathbf{CH_3}CH(OPO_3H^-)$-$COO^-$ with a dissociation constant of ~0.4 mM but do not bind $\mathbf{CF_3}CH(OPO_3H^-)COO^-$.[39]

Although MR binds TFL with an affinity similar to that exhibited for mandelate, the turnover numbers (k_{cat}) are markedly reduced by ~318-fold (Table 1). To compare the turnover of TFL with mandelate, it is important to bear in mind that superimposition of the carboxyl, hydroxyl, and α-proton of (*R*)-mandelate and (*S*)-TFL places the phenyl group of (*R*)-mandelate in the same spatial orientation as the CF_3 group of (*S*)-TFL (and vice versa). MR catalyzes the racemization of (*R*)-mandelate and (*S*)-mandelate with almost identical kinetic constants (Table 1); however, in general, MR catalyzes the racemization of (*R*)-mandelate with a slightly greater turnover number than exhibited for (*S*)-mandelate but binds the latter enantiomer slightly better.[14,20] MR exhibits the same enantioselectivity with TFL, catalyzing the racemization of (*S*)-TFL (cf. (*R*)-mandelate) with a greater turnover number than exhibited for (*R*)-TFL but binds the latter enantiomer slightly better.[14,20] Overall, MR catalyzes the racemization of TFL with efficiencies that are ~400-fold lower than those observed for mandelate (Table 1). This reduction in catalytic efficiency arises mainly from the reduction in the k_{cat} values. The value of K_{eq} (treating (*S*)-TFL as the product) calculated using the Haldane relation[40] is 0.9 ± 0.2, which is experimentally equal to unity, as expected for a racemase.

Because the reduction of the ellipticity observed during the assay could arise from MR-catalyzed β-elimination of fluoride, we conducted two experiments to demonstrate that MR was catalyzing the racemization of TFL. First, we observed that MR catalyzes the exchange of the α-proton with deuterium when the reaction was conducted in buffered D_2O (Figure 3) consistent with deprotonation−reprotonation events. Second, we used ^{19}F NMR spectroscopy to examine the products generated after "complete racemization" for evidence of the β-elimination product 3,3-difluoropyruvate. ^{19}F NMR spectra were obtained both for a reaction solution containing MR and (*S*)-TFL and for a control solution which lacked MR after each solution had been incubated for 16 h at 25 °C. During this time, the ellipticity of the reaction solution containing MR increased from −200.0 to −92.0 mdeg (at 228 nm), while the ellipticity of the control solution remained unchanged at −118.0 mdeg. (The observed ellipticity of the reaction solution does not go to zero upon completion of the reaction but attains a value of −92 mdeg due to the presence of MR and BSA in the solution.) The signal corresponding to the trifluoromethyl group appeared as a doublet at 0.08 ppm (J_{H-F} = 8.1 Hz), relative to the TFA internal standard, in the spectra of *both* the control and MR reaction samples (Figure 4). No fluorine signals corresponding to $CH\mathbf{F}_2$ or fluoride were detected in either spectrum. Hence, there was no evidence that β-elimination was catalyzed by MR when TFL was the substrate.

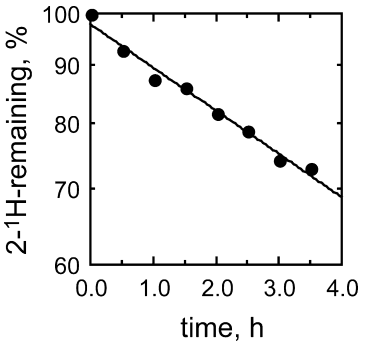


Figure 3. MR-catalyzed exchange of deuterium for the α-proton of (*S*)-TFL. The experimental conditions are as given in the Materials and Methods.

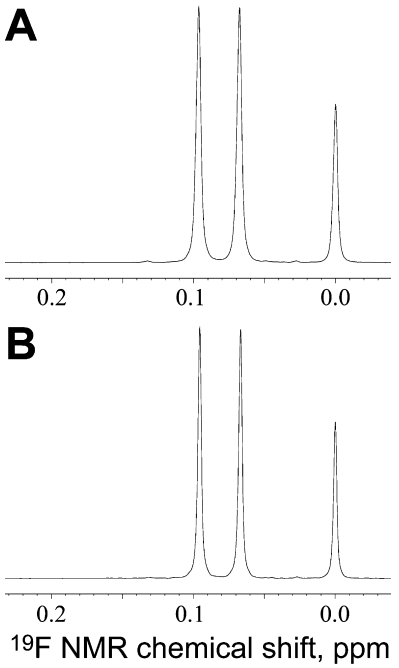


Figure 4. ^{19}F NMR spectra of the protein-free MR reaction solution obtained from the reaction of (*S*)-TFL (50 mM) (A) in absence of enzyme (i.e., control) and (B) in presence of MR (59 μg/mL) (i.e., MR reaction mixture) after incubation for 16 h in assay buffer at 25 °C. Chemical shifts are reported relative to TFA, which was employed as an internal standard.

Transition State Stabilization. The efficiency (k_{cat}/K_m) of an enzyme-catalyzed reaction and the rate constant for the corresponding nonenzymatic reaction (k_{non}) may be used to estimate an upper limit for the virtual dissociation constant

($K_{tx} = k_{non}/(k_{cat}/K_m)$) for the complex between the enzyme and the altered substrate in the transition state.[41−43] To determine the value of k_{non} (or k_{enant}) for the nonenzymatic racemization of TFL at 25 °C, we measured the first-order rate constants for the racemization of (*S*)-TFL at temperatures ranging between 120 and 170 °C. ^{19}F NMR analysis of samples after complete racemization revealed no indication of the formation of an elimination product (data not shown). Figure 5 shows an

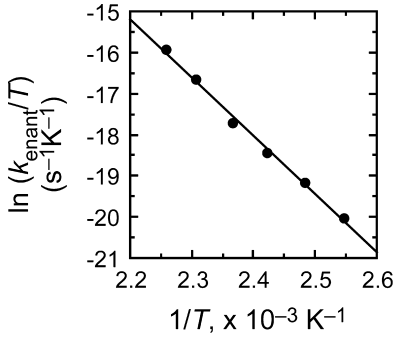


Figure 5. Effect of temperature on the observed first-order rate constant, k_{enant} (s^{-1}), for the uncatalyzed racemization of (*S*)-TFL at pH 7.5. Data points are the average of two independent determinations. The curve shown is the linear regression line [$\ln(k_{enant}/T) = (-14.1\ (\pm0.4) \times 10^3\ K)(1/T) + 15.8\ (\pm0.9)$] and yields values of 28.0 ± 0.7 kcal/mol and −15.7 ± 1.7 cal mol^{-1} K^{-1} for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, respectively.

Eyring plot of ln (k_{enant}/T) against $1/T$, which permits calculation of the activation parameters.[44] The values of the enthalpy of activation ($\Delta H^{\ddagger}$) and the entropy of activation ($\Delta S^{\ddagger}$) for the nonenzymatic racemization reaction are 28.0 (±0.7) kcal/mol and −15.7 (±1.7) cal K^{-1} mol^{-1}, respectively. Hence, the free energy of activation at 25 °C ($\Delta G^{\ddagger}_{25\,°C}$) is equal to 33 (±4) kcal/mol, corresponding to a nonenzymatic rate constant of ∼4 × 10^{-12} s^{-1} under assay conditions. Comparison of k_{non} with the turnover number for the MR-catalyzed racemization of (*S*)-TFL (k_{cat} = 2.5 s^{-1}) indicates a rate enhancement of (6.3 × 10^{11})-fold at pH 7.5 and 25 °C.

Comparison of k_{non} with the efficiency of MR-catalyzed racemization of (*S*)-TFL (k_{cat}/K_m = 1.4 × 10^3 M^{-1} s^{-1}) indicates that the upper limit for K_{tx} is ∼3 × 10^{-15} M. Hence, MR stabilizes the altered TFL in the transition state (ΔG_{tx}) by at least 20 kcal/mol (Figure 6). This is slightly less than the transition state stabilization of 26 kcal/mol afforded by MR when mandelate is the substrate (Figure 6).[29] Interestingly, the free energy of activation at 25 °C for the nonenzymatic racemization of mandelate (34.6 (±0.9) kcal/mol[29]) is experimentally equal to the free energy of activation for the nonenzymatic racemization of TFL. Even though the barriers for abstraction of the α-proton in the absence of the enzyme are essentially equivalent for mandelate and TFL, such is not the case for bound mandelate and TFL since their k_{cat} values differ by ∼318-fold. The acidifying effect of the phenyl ring of mandelate arises from both polar (inductive) effects and conjugation.[45] Since MR stabilizes the transition state for racemization of mandelate more effectively than it stabilizes the transition state for racemization of TFL by ∼6 kcal/mol, MR must interact with the phenyl ring of mandelate in the transition state to stabilize the negative charge more effectively through conjugation than can be achieved through inductive effects alone. For the enzymatic reaction, as opposed to the racemization of mandelate in solution, the binding of mandelate within the active site can enhance the acidity of the α-proton by enforcing a conformation of the phenyl ring that ensures efficient conjugation (see Figure 1). Such is not the case for TFL. Furthermore, MR may enhance conjugation through a double cation-π interaction as suggested by our previous finding that MR binds the phenyl ring of transition state analogues with an affinity that exceeds that predicted based on hydrophobic effects alone by ∼72-fold.[19] Although such interactions may also play a role in the enhanced ground state binding of TFL, the binding affinity of TFL only exceeds that predicted based on hydrophobic effects alone by ≤10-fold (vide supra). The enzyme's propensity to stabilize conjugation in the transition state is further illustrated by the fact that the value of k_{cat} for MR-catalyzed racemization of vinylglycolate (Scheme 1) is only reduced about 4-fold, relative to the value of k_{cat} for mandelate.[21]

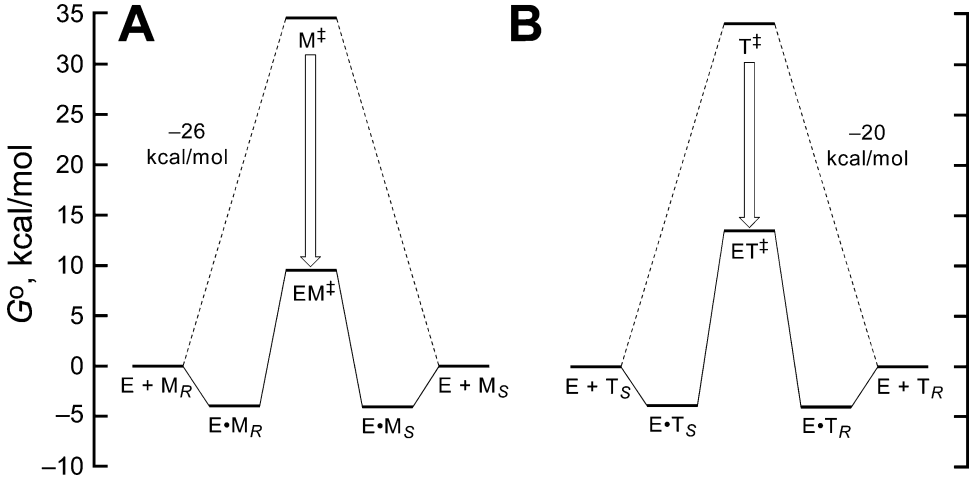


Figure 6. Free energy profile (pH 7.5, 25 °C) for the racemization of (A) (*R*)- (M_R) and (*S*)-mandelate (M_S) and (B) (*S*)- (T_S) and (*R*)-TFL (T_R) catalyzed by MR. The profiles are derived using the kinetic parameters given in Table 1. $T^{\ddagger}$ and $M^{\ddagger}$ represent the altered TFL and mandelate in the transition state, respectively, in the absence of enzyme. $ET^{\ddagger}$ and $EM^{\ddagger}$ represent the corresponding enzyme−substrate complexes in the transition state. The large downward-pointing arrows show the extent of transition state stabilization. Note that (*R*)-mandelate and (*S*)-TFL are compared because they share corresponding configurations about the α-carbon (see text). (Values are calculated for a standard state of 1 M.)

dx.doi.org/10.1021/bi201188j | *Biochemistry* 2011, 50, 8846−8852

Table 2. Effect of Viscosity on the Kinetic Parameters for MR-Catalyzed Racemization of (*S*)-TFLA

sucrose (%)	η/η^o	k_{cat} (s^{-1})	K_m (mM)	$k_{cat}{}^o/k_{cat}{}^\eta$	$(k_{cat}/K_m)^o/(k_{cat}/K_m)^\eta$
0.00	1.00	2.45 ± 0.25	1.74 ± 0.08	1.00 ± 0.14	1.00 ± 0.16
10.0	1.32	2.21 ± 0.43	1.71 ± 0.45	1.11 ± 0.24	1.09 ± 0.38
20.0	1.88	2.53 ± 0.46	1.64 ± 0.51	0.97 ± 0.20	0.92 ± 0.34
27.5	2.48	2.61 ± 0.19	1.49 ± 0.09	0.94 ± 0.12	0.80 ± 0.12
32.5	3.06	2.40 ± 0.05	1.53 ± 0.12	1.02 ± 0.11	0.90 ± 0.13
35.0	3.42	2.04 ± 0.26	1.42 ± 0.36	1.20 ± 0.19	0.98 ± 0.30

When mandelate is the substrate, both the chemical (proton abstraction) and product release steps are partially rate-determining as indicated by the partial viscosity dependence of MR reported previously.[20] Because MR is not as proficient at stabilizing the altered TFL in the transition state as it is at stabilizing the altered mandelate in the transition state, we examined the viscosity dependence of MR catalysis with (*S*)-TFL as the substrate using sucrose as the viscosogen (Table 2 and Figure 7). Indeed, no dependence on the microviscosity of the solvent was observed, consistent with the chemical step being rate-limiting.[46]

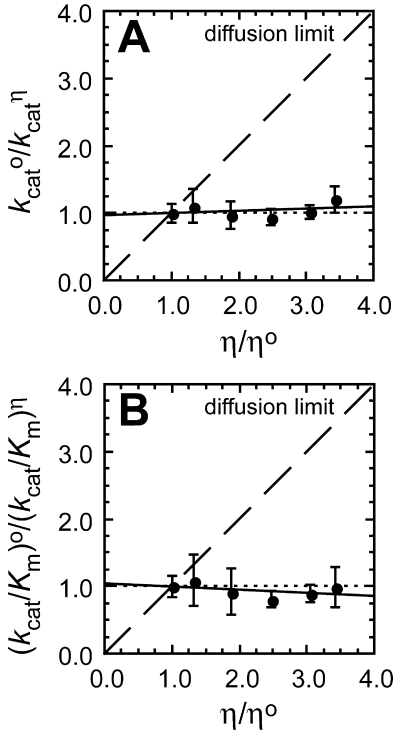


Figure 7. Dependence of relative kinetic parameters for the racemization of (*S*)-TFL on relative solvent viscosity. The (A) relative k_{cat} (i.e., $k_{cat}{}^o/k_{cat}{}^\eta$) values and (B) relative k_{cat}/K_m (i.e., $(k_{cat}/K_m)^o/(k_{cat}/K_m)^\eta$) values were determined at 25 °C at varying values of η_{rel} using sucrose as the viscosogen. The long-dashed line has slope = 1 and corresponds to the relative kinetic parameters expected when the rate of the reaction is fully limited by diffusion.

Thus, we have demonstrated that MR does not exhibit an absolute requirement for substrates having β,γ-unsaturation. Replacement of the phenyl ring of mandelate by the electron-withdrawing trifluoromethyl group is sufficient to permit MR to catalyze the racemization of TFL. However, MR catalyzes the racemization of this "activated" substrate with reduced catalytic proficiency relative to that observed for the racemization of substrates possessing β,γ-unsaturation.

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Funding

This work was supported by a Discovery Grant (S.L.B) and an Undergraduate Summer Research Award (Y.K.) from the Natural Sciences and Engineering Research Council (NSERC) of Canada.

ACKNOWLEDGMENTS

We express our thanks to Dr. Mike Lumsden (NMR-3) and Alexander C. Roy for their assistance in conducting the NMR experiments and to Jennifer Bourque for conducting some preliminary experiments.

ABBREVIATIONS

BSA, bovine serum albumin; CD, circular dichroism; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MR, mandelate racemase; TFA, trifluoroacetic acid; TFL, trifluorolactate.

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