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Inhibition of Mandelate Racemase by α-Fluorobenzylphosphonates

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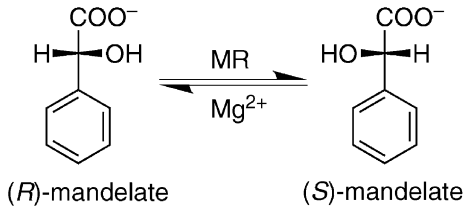
Abstract—Mandelate racemase catalyzes the interconversion of the enantiomers of mandelic acid. The enzyme binds the intermediate analogues (*R*)- and (*S*)-α-fluorobenzylphosphonate, and α,α-difluorobenzylphosphonate with 100–2500 times less affinity than it exhibits for (*R*,*S*)-α-hydroxybenzylphosphonate at pH 7.5. This apparent low affinity, relative to that of α-hydroxybenzylphosphonate, arises from the altered pK_a values of the α-fluorobenzylphosphonates. For example, (*S*)-α-fluorobenzylphosphonate is bound with the same affinity as the substrate at pH 7.5, but this affinity is increased ~6-fold at pH 6.3.

Mandelate racemase (E.C. 5.1.2.2; MR) from *Pseudomonas putida* catalyzes the Mg^{2+}-dependent 1,1-proton transfer which interconverts the enantiomers of mandelic acid (Scheme 1).[1] This 'pseudosymmetric' enzyme catalyzes the racemization of either substrate enantiomer with essentially identical kinetic parameters[2] and has been studied as a paradigm for enzymes which catalyze rapid carbon–hydrogen bond cleavage of carbon acids with relatively high pK_a values. Such enzyme-catalyzed reactions are common and understanding their mechanism is fundamental to delineating general principles of drug design.[3] MR catalysis proceeds via a two-base mechanism, with His 297 and Lys 166 abstracting the α-proton from (*R*)-mandelate and (*S*)-mandelate, respectively. Additional catalysis is provided by Glu 317 which acts as a general acid,[1] electrostatic interactions between Lys 164 and the carboxylate group of mandelate,[4] coordination of the substrate to the active site Mg^{2+} ion,[4,5] and H-bonding between the α-OH and Asn 197 (see Fig. 1).[6]



Scheme 1.

Our interest in understanding how protein-ligand interactions within the active site of MR stabilize the transition state for α-proton abstraction led us to survey a series of reactive intermediate analogues as potential transition state or reactive intermediate analogue inhibitors.[6] We identified α-hydroxybenzylphosphonate (α-HBP) and benzohydroxamate as potent reversible competitive inhibitors of MR. These analogues share geometric and electronic features with the putative *aci*-carboxylate intermediate and represent the most potent reversible inhibitors of this enzyme reported to date. Comparison of the observed inhibition constants for both carboxylate-containing and phosphonate-containing analogues indicated that a hydroxyl function located on a ligand's α-carbon is essential for potent inhibition. This observation, combined with the observation that the α-OH function is required for 1,1-proton transfer,[7] suggested that the α-OH function plays a significant role

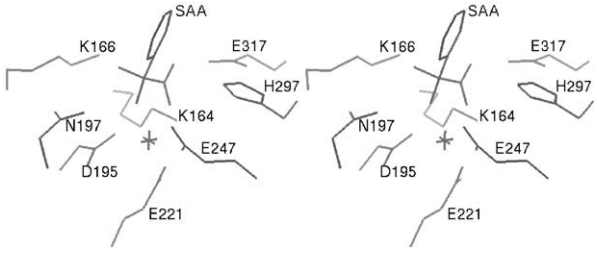


Figure 1. Stereogram of (*S*)-atrolactate (SAA; α-OH pointed toward Mg^{2+}) bound at the active site of wild-type MR (MDL).[7] The catalytic bases (Lys 166 and His 297), residues coordinating the magnesium ion (Asp 195, Glu 221, and Glu 247), and residues interacting with the ligand's carboxylate group (Lys 164 and Glu 317) and α-OH (Asn 197) are shown.

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in stabilizing the altered substrate in the transition state. Using site-directed mutagenesis to change Asn 197 to an alanine residue, we demonstrated that Asn 197 interacts with the α-OH function of mandelate to provide approximately 3.5 kcal/mol of transition state stabilization free energy to differentially stabilize the transition state relative to the ground state.[6]

To learn more about how variation of substituents on the α-carbon might affect inhibitor binding, we conducted a 'fluorinated phosphonate scan'[8] by evaluating the binding affinity of MR for the complete set of CF_2- and both stereoisomeric CHF-phosphonate derivatives of α-HBP. Unlike the α-OH function that can function both as an H-bond donor and acceptor, the α-fluoro group can only act as an H-bond acceptor. Increased polarization of the C_α-H bond caused by the presence of a fluorine atom on the α-carbon may permit additional interaction with the general base through H-bonding.

Chemistry and Enzymology

All reagents, with the exception of the benzylphosphonates, were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). The requisite α-fluorobenzylphosphonates (α-FBP)[9] and α,α-difluorobenzylphosphonate (α,α-F_2BP)[10] were prepared as described previously. Recombinant MR from *Pseudomonas putida* bearing an N-terminal hexahistidine tag was overexpressed, purified, and assayed using a circular dichroism (CD) assay as described previously.[11]

Inhibition assays containing either α,α-F_2BP (0.89–6.67 mM), (*R*,*S*)-α-FBP (0.22–1.78 mM), (*R*)-α-FBP (0.17–0.713 mM), or (*S*)-α-FBP (0.11–0.67 mM) at the concentrations indicated, were conducted at 25 °C in Hepes buffer (0.1 M, pH 7.5) or Mes buffer (0.1 M, pH 6.3) each containing $MgCl_2$ (3.3 mM), and wild-type MR (150 ng/mL). Substrate ((*R*)-mandelate) concentrations typically ranged between 0.22 and 9.50 mM. Competitive inhibition constants (K_i) were determined from plots of the apparent K_m/V_{max} values versus inhibitor concentration as described previously.[11]

Results and Discussion

Hydrogen bonding often plays an important role in enzyme-substrate interactions. In fact, the X-ray crystal structure of MR with the bound substrate analogue, (*S*)-atrolactate (Fig. 1), reveals the potential for multiple hydrogen bonds between the protein and the ligand.[4,7] The importance of these polar interactions is underscored by the major enthalpic contribution that they make to transition state stabilization.[12] Fluorine is often used as an isosteric replacement for OH groups.[13] Because a fluorine substituent cannot donate an H-bond but can only act as an H-bond acceptor, substitution of the α-OH on the ligand by a fluorine restricts the H-bonding capability of that ligand. For example, deoxyfluoro sugars have been widely employed in studies of binding interactions between enzymes and their carbohydrate substrates.[14] In addition to restricting the H-bonding interactions, replacement of the α-OH of α-HBP by the highly electronegative fluorine atom would be expected to alter the properties of the benzylphosphonate ligand in three ways. First, the substitution of fluorine atoms on the α-methylene of phosphonates lowers the pK_a of the phosphonate.[15,16] Second, the presence of the fluorine polarizes the C_α–H bond so that it may act as an H-bond donor.[17] Third, coordination of the active site Mg^{2+} ion by the α-OH is lost.

The fluorinated analogues of the intermediate analogue inhibitor α-HBP ((*R*,*S*)-, (*R*)-, and (*S*)-α-FBP, and α,α-F_2BP) were all competitive inhibitors of MR. The values of the inhibition constants are given in Table 1 and a representative double reciprocal plot is shown in Figure 2. MR binds the various α-FBPs with approximately 100–2500 times *less* affinity than it exhibits for (*R*,*S*)-α-HBP. Interestingly, MR displayed a slightly higher affinity for (*S*)-α-FBP than for the corresponding (*R*)-enantiomer. This observation is consistent with our previous observation that MR binds (*S*)-α-HBP with an

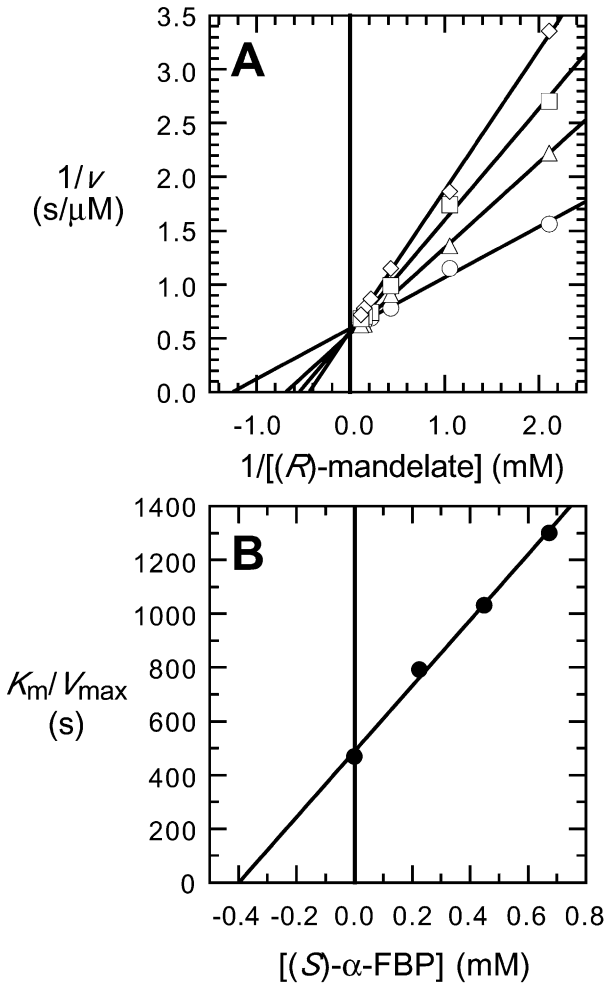
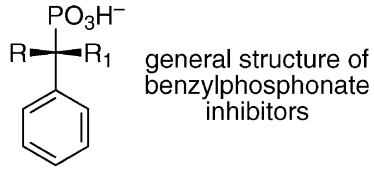


Figure 2. Inhibition of MR by (*S*)-α-FBP. A representative double-reciprocal plot is shown in panel A. Assays were conducted as described in the text in the presence of 0 mM (○), 0.224 mM (△), 0.447 mM (□) and 0.671 mM (◇) of (*S*)-α-FBP. A plot of the reciprocal slopes obtained from the initial velocity curves as a function of (*S*)-α-FBP concentration is shown in panel B.

Table 1. Inhibition of MR by phosphonate-containing ligands



Ligands	R	R_1	pK_{a2} of the phosphonate	K_i[a] (mM)
(*R*)-mandelate[b]	—	—		0.59 (±0.08)
(*S*)-mandelate[b]	—	—		0.57 (±0.07)
(*R*,*S*)-α-HBP	OH/H	H/OH	6.89[c]	0.0047 (±0.0007)[c]
(*R*)-α-HBP	OH	H	6.89[c]	0.034 (±0.009)[c]
(*S*)-α-HBP	H	OH	6.89[c]	0.0011 (±0.0007)[c]
benzylphosphonate	H	H	7.6[d],7.72[e]	3.6 (±0.7)[c]
benzoylphosphonate	=O	—	5.46±0.02[f]	0.30 (±0.06)[c]
α,α-F_2BP (pH 7.5)	F	F	5.71[e]	12 (±4)
(pH 6.3)				1.0 (±0.2)
(*R*,*S*)-α-FBP	F/H	H/F	6.5[d],6.60[e]	0.81 (±0.34)
(*R*)-α-FBP	F	H	6.5[d],6.60[e]	1.11 (±0.45)
(*S*)-α-FBP (pH 7.5)	H	F	6.5[d],6.60[e]	0.53 (±0.17)
(pH 6.3)				0.087 (±0.012)

[a]Values are means of three experiments conducted at pH 7.5 unless indicated otherwise. Standard deviation is given in parentheses.
[b]K_S values from ref 12.
[c]K_i and/or pK_a values from ref 6.
[d]pK_a values from ref 15.
[e]pK_a values from ref 18.
[f]pK_a determined in the present study using titration.

affinity that is 30-40-fold greater than that observed for (*R*)-α-HBP,[6] unmasking a 'functional asymmetry' within the active site. Incubation of (*R*)-α-FBP (9.6 mM) with mandelate racemase (30 μg/mL) for 25 h under the assay conditions did not produce any change in the observed ellipticity indicating that (*R*)-α-FBP is not a substrate for mandelate racemase.

In previous studies, we examined the pH-dependence of inhibition by α-HBP and showed that MR preferentially binds the monoanionic form of α-HBP.[6] Examination of the pK_a values for the phosphonates in Table 1 suggests that the lower binding affinity results, in part, because fluorines on the α-carbon lower the pK_a of the phosphonate function such that less of the FBPs exist in solution as the monoanions (e.g., the percentages of (*S*)-α-HBP, (*S*)-α-FBP, and α,α-F_2BP present as the monoanionic species at the assay pH of 7.5 are approximately 20%, 10%, and 1.6%, respectively). Indeed, the enzyme's affinity for both (*S*)-α-FBP and α,α-F_2BP is enhanced 6- and 12-fold, respectively, when the assay pH is reduced from 7.5 to 6.3. The same change in assay pH, however, does not significantly alter the enzyme's affinity for the substrate (i.e., K_m ($=K_S$) = 0.93±0.15 mM at pH 7.5 and K_m = 0.79±0.13 mM at pH 6.3).

Comparison of the pK_a values for (*S*)-α-HBP and (*S*)-α-FBP indicates that the proportion of monanionic species present in solution is only reduced 2-fold by the presence of the fluorine yet the binding affinity is reduced 482-fold. This implies that additional binding interactions are being lost when the α-OH of α-HBP is replaced by a fluorine atom. The most obvious interaction that is lost is coordination of the Mg^{2+} ion by the α-OH. It is possible to estimate the free energy associated with Mg^{2+} coordination using the equilibria shown in Figure 3.

Comparison of the stability constants for the chelation of divalent metal ions (Zn^{2+} and Cu^{2+}) by α-ketobutanoic acid as compared to 2-hydroxybutanoic acid and by acetoacetate as compared to 3-hydroxybutanoic acid

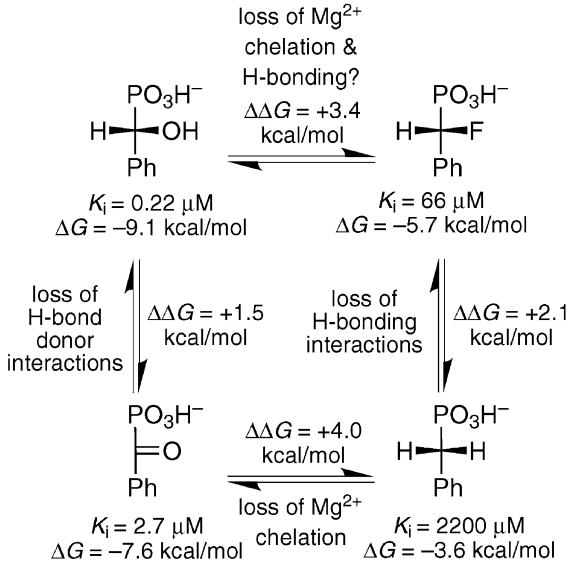


Figure 3. Thermodynamic cycle showing the apparent free energy changes associated with the loss of Mg^{2+} chelation and H-bonding interactions. The K_i value for each inhibitor has been adjusted based on the corresponding phosphonate pK_a value (Table 1) to reflect the concentration phosphonate monoanion present at pH 7.5. Free energy changes were calculated using the equation $\Delta\Delta G = -RT\ln(K_i^A/K_i^B)$ where A and B might represent α-HBP and α-FBP, respectively. (Note that the K_i value for (*S*)-HBP is for a substance with 80% ee.[6])

indicates that α-hydroxy acids tend to form only slightly stronger chelates than the corresponding keto acids by factors ranging between 1.1 and 3.3.[19] Hence, both α-HBP and benzoylphosphonate should chelate Mg^{2+} to approximately the same extent. In addition, the H-bond donating ability of the α-OH would be lost and the carbonyl oxygen would be expected to act only as a weak H-bond acceptor because of its coordination to Mg^{2+}. This implies that the free energy change associated with replacement of the α-OH by a carbonyl function (i.e., α-HBP→benzoylphosphonate) should reflect the elimination of virtually all H-bonding interactions between the α-position and the enzyme (i.e., $\Delta\Delta G = +1.5$ kcal/mol). (^{31}P NMR analysis (101.26 MHz, ^{1}H decoupled) of benzoylphosphonate (0.1 M in D_2O, pD 7.5) revealed the presence of only a single peak (δ 2.39 ppm relative to external phosphoric acid) that most likely corresponds to the nonhydrated dianion. O'Brien et al.[20] reported that ~92% of methyl acetylphosphonate exists as the nonhydrated form between pH 3 and 10. Hence, it is unlikely that a significant proportion of the benzoylphosphonate monoanion would be hydrated.) The free energy change associated with replacement of the carbonyl function with hydrogen (i.e., benzoylphosphonate→benzylphosphonate), on the other hand, will largely reflect the loss of Mg^{2+} coordination by the α-OH (i.e., $\Delta\Delta G = +4.0$ kcal/mol).

The free energy change associated with replacing the α-OH by an α-F (i.e., α-HBP→α-FBP) is difficult to interpret because it could represent the loss of an H-bond if the α-OH is an H-bond donor and loss of Mg^{2+} coordination. In addition, a new H-bonding interaction between the α-H of α-FBP and the general base that is responsible for abstraction of that proton during catalysis could be present. (Note that the C_α-F bond in α,α-F_2BP corresponding to the C_α–H bond in (*S*)-α-FBP could accept an H-bond from the conjugate acid of the enzymic general base.) Likewise, the free energy change associated with replacing the α-F by hydrogen (i.e., α-FBP→benzylphosphonate) is also difficult to interpret. It could represent the loss of an H-bond interaction between the α-F and an H-bond donor within the active site and/or the loss of the H-bond donating ability of the C_α–H in α-FBP. Despite our inability to delineate the precise interactions between the enzyme and α-FBP, it is clear that, after accounting for the loss of ~3.4–4.0 kcal/mol due to the loss of Mg^{2+} coordination, there remains ~2 kcal/mol of binding free energy that the presence of fluorine affords to the inhibitor, relative to benzylphosphonate.

Thus replacement of the α-OH of α-HBP by fluorine yields an inhibitor for which the monoanion binds approximately 10-fold more tightly than the substrate. In addition, it appears that MR may use interactions to bind α-fluorobenzylphosphonates that are different from those that it uses to bind α-HBP. With respect to inhibitor design, it is apparent from this work that the effect of replacing an OH by F is not simply limited to restricting the H-bond donating ability of the OH group, but also depends on other local interactions within a binding site.

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