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Mechanism-based Inactivation of Mandelate Racemase by Propargylglycolate

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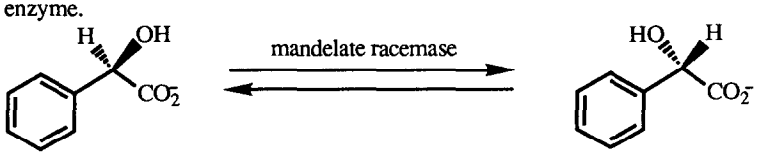
Abstract: Propargylglycolate (2-hydroxy-3-butynoic acid) has been determined to be both a substrate and an inactivator of mandelate racemase. The partition ratio for racemization/inactivation has been estimated to be ~17,000. Inactivation of the racemase appears to require the rapid covalent addition of 1 substrate molecule; however, a slower labeling process is observed that results in the attachment of up to 5 molecules of substrate per active site. The process is consistent with an enzyme-catalyzed rearrangement of the acetylenic substrate to an allenic-enol that affords 2-keto-3-butenoate as the ultimate electrophile.

Mandelate racemase (MR) (EC 5.1.2.2) from *Pseudomonas putida* ATCC 12633 catalyzes the interconversion of the (*R*)- and (*S*)-enantiomers of mandelic acid (Scheme 1). The enzyme is Mg^{2+}-dependent and the reaction proceeds via an abstraction of the α-proton from either enantiomer of mandelate followed by a stereorandom reprotonation of the stabilized enol(ate) intermediate. Kinetic and structural evidence has suggested the occurrence of two distinct acid/bases in the active site of MR: a histidine residue (H297) effecting proton abstraction/addition from the (*R*)-face of mandelate and a lysine residue (K166) operating from the (*S*)-face.[1-3]

While the racemase exhibits a broad substrate specificity with respect to substitutions on the aromatic nucleus of mandelate,[4,5] α-hydroxyalkanoic acids are not substrates. For example, lactate and 2-hydroxybutanoic acid do not undergo racemization by the enzyme. During our search for alternate substrates for the racemase, we discovered that vinylglycolate (2-hydroxy-3-butenoic acid) is an excellent substrate with kinetic parameters comparable to mandelate.[6] In addition, the enzyme was found to slowly convert vinylglycolate to α-ketobutyrate by an enzyme-catalyzed allylic rearrangement to the enol of α-ketobutyrate followed by tautomerization. This side-reaction occurred at a rate ~10^{-4} that of racemization.

In this report we present our preliminary findings on the reaction of mandelate racemase with propargylglycolate (2-hydroxy-3-butynoic acid). The results are consistent with the facile racemization of this α-hydroxy acid and a slow rearrangement to an electrophilic species which effects covalent modification and inactivation of the enzyme.



Scheme 1. Reaction catalyzed by mandelate racemase.

RESULTS

Inactivation of mandelate racemase by propargylglycolate

Propargylglycolate was found to be an irreversible inactivator of mandelate racemase. The semi-log plot of remaining enzyme activity versus time of preincubation in the presence of (*R,S*)-propargylglycolate is shown in Figure 1. The values of $t_{1/2}$ obtained from this plot were used to calculate $k_{inact}(obs)$ for each concentration of the inhibitor. A replot of these data yielded the following kinetic constants: $k_{inact} = 0.28 \pm 0.02$ min^{-1} and $K_I = 0.37 \pm 0.02$ mM. The kinetic constants obtained with (*S*)-propargylglycolate were similar to the values for the racemic mixture. Figure 1 also demonstrates that the inactivation displays protection by substrate and by the addition of exogenous thiols (5 mM ß-mercaptoethanol).

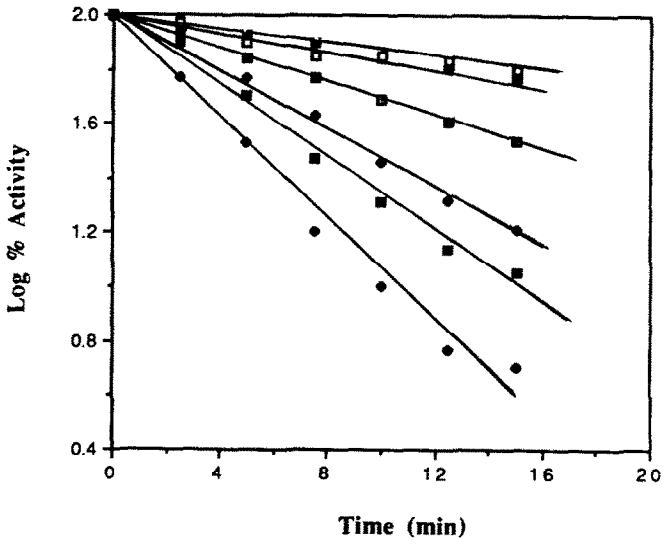


Figure 1. Kinetics of irreversible inactivation of mandelate racemase by (*R,S*)-propargylglycolate. The concentrations of compound used were 1 mM + 2 mM racemic mandelate (solid squares), 1 mM + 5mM ß-mercaptoethanol (hollow squares), 0.125 mM (closed squares), 0.25 mM (solid diamonds), 0.5 mM (closed squares, and 1 mM (hollow diamonds).

The kinetics of inactivation were also investigated in D_2O. The kinetic values were determined to be $k_{inact} = 0.20 \pm 0.01$ min^{-1} and $K_I = 11.6 \pm 0.44$ mM. These values give a $^D(V)$ isotope effect of ~1.4 and a $^D(V/K)$ effect of ~44 on the inactivation process. The unusually large selection effect is probably indicative of the low commitment to inactivation and the multiple proton transfer steps involved in the inactivation process (vide infra).

Racemization of progylglycolate by mandelate racemase

The resolved propargylglycolates were tested as substrates for the racemase. The reciprocal plots [1/v (μmol·min^{-1}) versus 1/[S] (mM^{-1})] for the racemization of (*R*)- and (*S*)-pargylglycolates in H_2O and of (*S*)-propargylglycolate in D_2O are shown in Figure 2. In H_2O the values obtained for k_{cat} and K_m for the (*S*)-isomer are 79 s^{-1} and 4.13 mM, respectively; for the (*R*)-isomer the values are 37 s^{-1} and 2.9 mM. In D_2O the values obtained for (*S*)-propargylglycolate were 20 s^{-1} and 3.9 mM. Using the above values, a partitioning of propargylglycolate between racemization and inactivation may be calculated. In H_2O $^{H}(k_{cat}/k_{inact})$ equals 17,200, while in D_2O $^{D}(k_{cat}/k_{inact})$ equals 6,000.

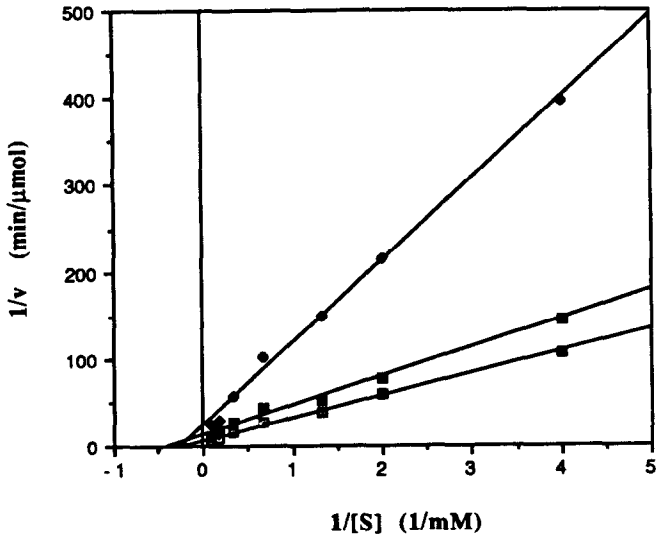


Figure 2. Lineweaver-Burk plot of the racemization of propargylglycolate catalyzed by mandelate racemase. (*R*)-Propargylglycolate in H_2O (closed squares); (*S*)-propargylglycolate in H_2O (open squares); (*S*)-propargylglycolate in D_2O (solid diamonds).

The racemization by proton abstraction/addition was verified by monitoring the exchange of the α-proton of the (*S*)-isomer in D_2O by ^1H NMR (Figure 3). A time-dependent change in the spectrum was observed. In the starting compound the α-proton (δ 4.55 ppm) is coupled (J = 2.5 Hz) to the acetylenic proton (δ 2.65). In the presence of the racemase, the signal corresponding to the α-proton decayed in a time-dependent manner and the acetylenic proton resonance collapsed to a singlet. The kinetics of this process were similar to those observed in the CD assay. This result constitutes a direct observation of the enzyme-catalyzed exchange of the α-proton with solvent deuterium.

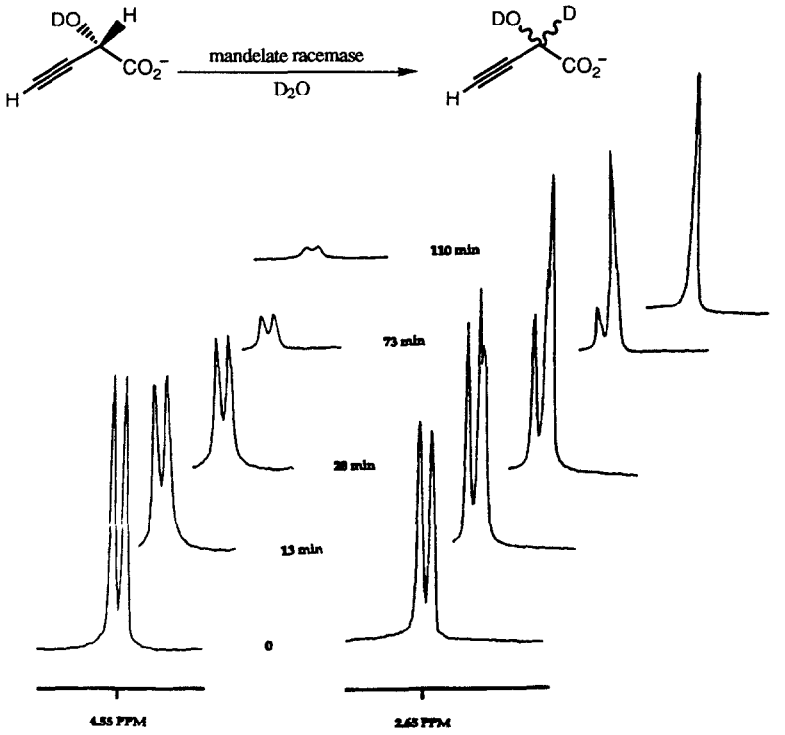


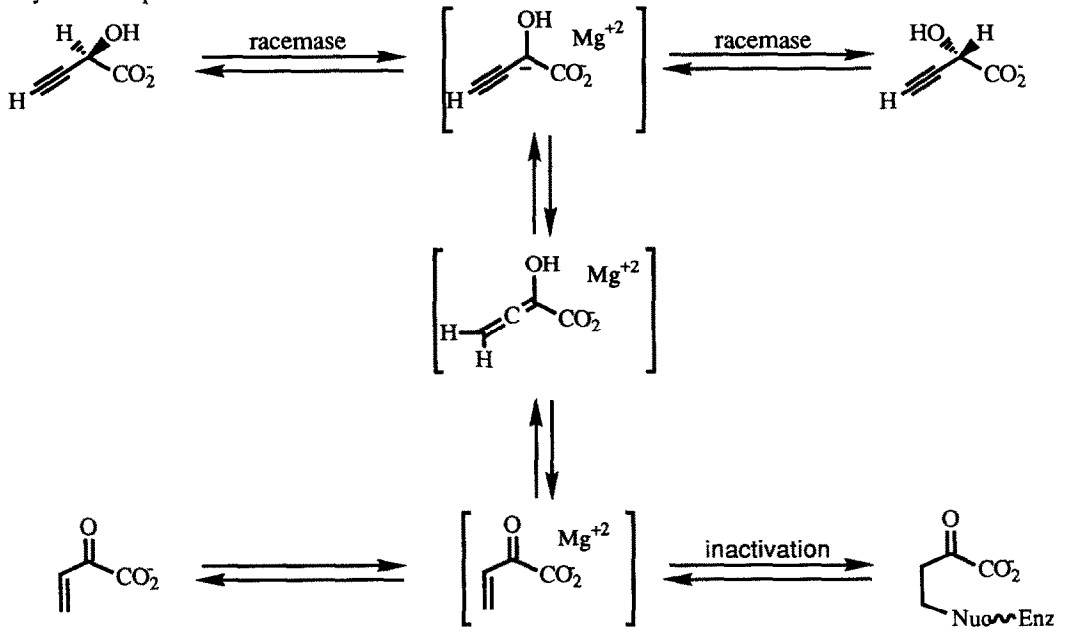
Figure 3. Time-dependent change in the ^1H NMR spectrum of (*S*)-propargylglycolate in the presence of mandelate racemase. At time zero, 10 units of racemase were added to a solution of 28 mM (*S*)-propargylglycolate in 20 mM HEPES and 1 mM $MgCl_2$, pD 7.5 (25 °C).

Covalent labeling of mandelate racemase by [1-^{14}C]-(R,S)-propargylglycolate

When incubated with [1-^{14}C]-(*R,S*)-propargylglycolate, mandelate racemase was found to incorporate 5.1 moles of label/active site. This extent of incorporation required ~20 h to reach completion. However, under the conditions employed, racemase activity was nearly completly lost (7 % of original activity) in 10 min after the incorporation of ~0.8 moles label/mole active site. This finding suggests that, while a single, presumably active site specific, covalent modification results in inactivation of the enzyme, a significant amount of the reactive intermediate is released into solution where it is available to slowly react with other nonessential nucleophiles on the inactivated enzyme. The possibility that the reactive intermediate is released from the enzyme is corroborated by the observation that ß-mercaptoethanol partially protects the enzyme from inactivation (Figure 1).

DISCUSSION

Our findings are consistent with the proposal outlined in Scheme 2. As a substrate analog, propargylglycolate undergoes a rapid (k_{cat} = 40 - 80 s^{-1}) racemization catalyzed by the enzyme. On the basis of our previous studies,[1-3] this process occurs via α-proton abstraction/readdition by stereofacially distinct, active site acid/bases. An enzyme stabilized enol(ate) has been proposed to be a kinetically competent intermediate.[3] By analogy to our recent observations on the racemase-catalyzed isomerization of vinylglycolate,[6] we propose that the enol(ate) undergoes a slow, racemase-catalyzed rearrangement to afford the allenic-enol. The rate of formation of the allene must be faster than the rate of inactivation of the racemase (k_{inact} = 0.2 - 0.3 min^{-1}); however, it is also considerably slower than the rate of racemization. Although the allenic-enol could potentially serve as the reactive electrophile leading to inactivation, we favor its facile ketonization to 2-keto-3-butenoate. This electrophile can react with an active site nucleophile via a Michael-type conjugate addition resulting in covalent modification and inactivation of the racemase. The multiple covalent modifications of the racemase (5 per monomer) which occur long after inactivation of the racemase suggest that the rate of release of the 2-keto-3-butenoate from the active site is considerably faster than the rate of inactivation. The free electrophile could then react with nonessential enzyme nucleophiles.



Scheme 2. Proposed mechanism of inactivation of mandelate racemase by propargylglycolate.

Propargylglycolate has been demonstrated to be a mechanism-based inactivator of a number of lactate oxidases and dehydrogenases containing the redox cofactor flavin mononucleotide (FMN).[7-10] In these cases, two pathways have been proposed which can lead to inactivation: 1) nucleophilic attack of the carbanionic intermediate, generated by α-proton abstraction, on the oxidized flavin; or 2) oxidation of the hydroxy group by flavin to give 2-keto-3-butynoate which adds electrophilically to the reduced flavin. A definitive distinction between these mechanisms has yet to be made. The inactivation of D-amino acid oxidase by D-propargylglycine also bears some similarity to our work.[11] Inactivation is believed to occur via the allene, 2-keto-3,4-pentadienoate, which was postulated to arise from the initial product 2-keto-4-pentynoate by a facile rearrangement.

Our proposal predicts that 2-keto-3-butenoate is an alternate product of the racemase, although it is generated is low amounts. Our initial attempts to detect this compound by trapping procedures have not been successful; however, on the basis of our studies with vinylglycolate,[6] we believe that this is a reflection of its reactivity and the small amounts formed.

In conclusion, our findings demonstrate that propargylglycolate is a novel substrate/ inactivator of mandelate racemase. Thus, the racemase can catalyze the α-proton abstraction/addition of activated, non-aromatic α-hydroxy acids. This observation may be of use in elaborating the mechanistic details of the reaction and in understanding the factors important in enol(ate) stabilization by the enzyme.

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EXPERIMENTAL

Syntheses of propargylglycolate

All chemicals (Aldrich) used in synthetic procedures were reagent grade or better. Where indicated, solvents were dried using standard. Propargylaldehyde was prepared by chromium trioxide oxidation of propargyl alcohol.[12] (*R,S*)-Propargylglycolate (2-hydroxy-3-butynoic acid) was prepared by acid hydrolysis of the trimethylsilyl ether formed between propargylaldehyde and trimethylsilyl cyanide following the general procedure of Gassman and Talley.[13] (*R*)- and (*S*)-Propargylglycolate were resolved from the racemic mixture by a modification of the procedure of Fendrich and Ghilsa.[14] (*S*)-Isomer: $[\alpha]_D^{25} = +31^o$ (*c* 0.1, H_2O) (lit.[14] $[\alpha]_D^{25} = +29^o$ [*c* 0.4, H_2O]); (*R*)-isomer: $[\alpha]_D^{25} = -24^o$ (*c* 0.1, H_2O). [1-^{14}C]-(*R,S*)-Propargylgylcolate was prepared with [^{14}C]NaCN by the procedure of Pompon and Lederer[15] in 20% yield (based on initial radioactivity; specific activity = 6 x 10^6 cpm/μmol).

Purification of mandelate racemase

Wild-type mandelate racemase was purified by the method of Tsou et al.[16] The specific activity was 1,010 U/mg (k_{cat} ~700 s^{-1}; ~39 kDa/monomer) as determined by the circular dichroic assay[17] in 100 mM HEPES, pH 7.5, containing 5 mM (*R*)-mandelate and 3 mM $MgCl_2$, at 25 °C. The concentration of the enzyme stock solution was determined using the micro-biuret assay[18] with bovine serum albumin as a standard.

Inhibition of mandelate racemase by propargylglycolate

The inhibition of mandelate racemase with propargylglycolate was performed according to a modification of the protocol of Fee et al.[19] The enzyme was incubated with varying concentrations of propargylglycolate in 100 mM HEPES, pH 7.5. Residual enzyme activity was assessed at specific time points by the circular dichroic assay[6] via addition of 5 mM (*R*)-mandelate.

Racemization of propargylglycolate

The racemization of (*R*)- and (*S*)-propargylglycolates were determined by the CD assay at 217 nm, as described above. At 217 nm, [θ] equals 1,720 deg·cm^2·dmol for the (*S*)-isomer while [q] equals 1,420 deg·cm^2·dmol for the (*R*)-isomer. This difference is presumably due to contamination of the (*R*)-isomer with the (*S*)-isomer.

^1H NMR analysis of the racemization was performed on an IBM AF 400 (FT) spectrometer. At time zero, 10 units of mandelate racemase were added to a solution of 28 mM (S)-propargylglycolate in 20 mM HEPES and 1 mM $MgCl_2$, pD 7.5 (25 °C).

Covalent labeling of mandelate racemase by [1-^{14}C]-(R,S)-propargylgylcolate

Mandelate racemase (0.01 μmol active sites) and [1-^{14}C]-(*R,S*)-propargylgylcolate (5.3 mM final concentration, 1.5 x 106 cpm/mol) were incubated in HEPES-Mg buffer, pH 7.5 (1 mL final volume). At various times, 50 μL aliquots were removed and the excess inactivator was separated from enzyme by the centrifuge column technique. The stoichiometry of labeling was determined by liquid scintillation counting of a 30 μL aliquot of the centrifuged sample. The residual enzyme activity was determined on a 20 μL portion of the sample by the CD assay.

Control experiments were performed to ensure that free inactivator was completely adsorbed in the centrifuge column matrix and any radioactivity eluting with protein was due to enzyme-bound inhibitor. Heat-denatured racemase that was incubated with the radiolabeled inactivator as described above was shown to be devoid of radioactivity subsequent to removal of excess inactivator.

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