Mandelate Racemase: Structure–Function Studies of a Pseudosymmetric Enzyme

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Introduction

Racemases (and their close cousins, the epimerases) have always intrigued biochemists because they must process both substrate enantiomers (or diastereoisomers in the case of epimerases). So the question arises, how can enzymes, which are inherently asymmetric, deal with both enantiomers with at least approximately equal facility? Most enzymes, after all, are famous for being exquisitely stereoselective. But racemases by definition have equilibrium constants equal to unity and therefore, according to the Haldane relationship, k_{cat}/K_m in one direction must be equal to k_{cat}/K_m in the reverse direction. The logical answer to this question is that racemases must have evolved a "functional asymmetry" or "pseudosymmetry" in their active sites.[1–4] In this Account, we provide considerable insight into this question as we now have an X-ray crystal structure of mandelate racemase and several of its mutational variants at reasonably high resolution.[5–14] In fact, mandelate racemase is so far the only racemase for which an X-ray crystal structure is available.

Another puzzling mechanistic question in enzymology is, how can enzymes catalyze rapid proton exchange (and racemizations, isomerizations, or β-eliminations) involving carbon–hydrogen bond cleavage of carbon acids with relatively high pK_a values? The pK_a for the α-hydrogen of mandelic acid, for example, has been estimated to be ~22 and that for the mandelate anion to be ~29.[15] The difficulty in removing this proton has been shown experimentally by Pocker,[16] who found that sodium mandelate undergoes exchange of its α-hydrogen in 0.40 M NaOD in D_2O only very slowly even at 100 °C. In contrast, enzymatically, this same exchange reaction occurs with a turnover number of ~1000 s^{-1} at 25 °C even at pH 7.[17] In this Account, some important insights will be presented

George L. Kenyon was born in Wilmington, DE, in 1939. He received his B.S. in chemistry from Bucknell and his A.M. and Ph.D. degrees in organic chemistry from Harvard, working with Professor F. H. Westheimer. After an NIH postdoctoral fellowship in biochemistry at MIT with Professor J. M. Buchanan (1965–1966), he joined the faculty at the University of California, Berkeley, as assistant professor of chemistry. He moved to UCSF in 1972, where he is currently professor of chemistry and pharmaceutical chemistry and dean of the School of Pharmacy. He has wide-ranging research interests that include studies of the mechanisms of enzymatic reactions, applications of recombinant DNA technology to enzymology, and the development of structure-based approaches to the rational design of both enzymatic inhibitors and nucleic acid binding agents as potential drugs. With his wife, Lucy, he also enjoys the observations of avifauna on a global scale, and in this regard, he has been known at times to be considered a "twitcher".

John A. Gerlt was born in Sycamore, IL, in 1947. He earned his B.S. degree in biochemistry from Michigan State University (1969) and his A.M. (1970) and Ph.D. (1974) degrees in biochemistry and molecular biology from Harvard University, where he studied with Professor Frank H. Westheimer. His postdoctoral studies, supported by a Jane Coffin Childs Memorial Fund Fellowship, were with Dr. Christian B. Anfinsen at the National Institutes of Health, Bethesda, MD (1974–1975). He has held positions as assistant and associate professor of chemistry at Yale University (1975–1984) and as professor of chemistry and biochemistry at the University of Maryland, College Park (1984–1994). He recently moved to the University of Illinois, Urbana-Champaign, where he is professor and head of biochemistry. His research interests are focused on understanding the relationships among rates, mechanisms, and active site architectures of various enzyme-catalyzed reactions. His spare time is spent with his wife Jennifer enjoying salt water fish, both in his tank at home and on the coral reefs of St. John. In addition to the fish, his pets include a dog named Eminence and cats named Frank and George.

Gregory A. Petsko was born in Washington, DC, in 1948. He received his A.B. from Princeton University and his D.Phil. from Oxford University, where he did his thesis research with Sir David Phillips. After a brief stint with Professor Pierre Douzou in Paris, he joined the faculty of Wayne State University School of Medicine as instructor in biochemistry, becoming assistant professor in 1976. He moved to MIT in 1979 as associate professor and later (1985) professor of chemistry. In 1990 he moved to Brandeis University, where he is now Lucille P. Markey Professor of Biochemistry and Chemistry and director of the Rosenstiel Basic Medical Sciences Research Center. His research interests include the structural basis of the catalytic power of enzymes, the functional roles of protein dynamics, hyperthermostability, and the structural enzymology of mRNA turnover. His nonscientific diversions include old books, old movies, old cars, and old Scotch.

John W. Kozarich is executive director of biochemistry at Merck Research Laboratories in Rahway, NJ. After graduation from Boston College (1971), he completed his Ph.D. research in biological chemistry at MIT with Sidney Hecht (1975) and did a postdoctoral in biochemistry at Harvard with Jack Strominger. He has been professor of pharmacology at Yale University School of Medicine and professor of chemistry and biochemistry at the University of Maryland, College Park. An American Cancer Society Faculty Research Award (1983–1988) and the Pfizer Award in Enzyme Chemistry (1988) are his most notable recognitions. His research interests are mechanisms of enzyme and drug action and their application to drug discovery and enzyme catalysis.

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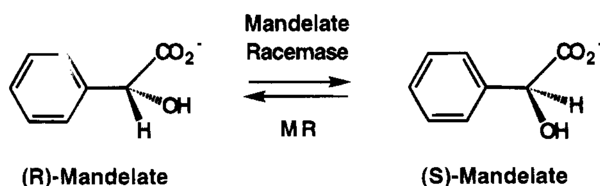
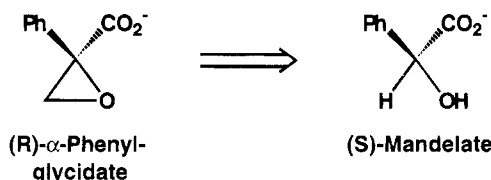
Scheme 1. Reaction Catalyzed by MR



Scheme 2. Stereochemical Relationship between (*R*)-α-Phenylglycidate and (*S*)-Mandelate



concerning this mechanistic puzzle, insights that should be generalizable to a host of related enzymatic reactions.

Mandelate racemase (MR, EC 5.1.2.2) catalyzes the reversible interconversion of the (*R*)- and (*S*)-enantiomers of mandelate (Scheme 1). This Account will focus on mechanistic findings that have been made by our research consortium over the past decade. Early work, which has been reviewed previously,[18] established that the enzyme contains no organic cofactors and showed that stabilization of negative charge on the carbon α to the carboxylate (by using electron-withdrawing substituents in the para position of the phenyl ring) accelerates the enzymatic racemization.[19] Also, the enzyme shows a primary deuterium isotope effect of ~3 in both directions when [α-^{2}H]mandelates were used as substrates.[3,20] These observations, as well as the observation that the enzyme catalyzes carbon–hydrogen isotopic exchange that accompanies racemization, led to the early hypothesis that the enzyme functions by using acid–base chemistry to promote carbon–α-hydrogen bond cleavage as its fundamental mechanistic strategy.[20,21] These studies were followed by reports that demonstrated conclusively that MR has an absolute divalent metal ion requirement with Mg^{2+} as the most effective followed by Co^{2+}, Ni^{2+}, Mn^{2+}, and Fe^{2+} in that order.[22] This absolute divalent metal ion requirement even extends to the affinity labeling of the enzyme by α-phenylglycidate (Scheme 2).[23,24] The required divalent metal ion was found to be tightly bound to MR and close to the bound mandelate, suggesting that the metal ion (Mn^{2+} in these experiments) helps weaken the scissile bond by its proximity to the substrate's carboxylate group.[17]

Mandelate Racemase: Biological Context, Cloning, and Overexpression

Mandelate racemase is part of the mandelate pathway in *Pseudomonas putida*, which is composed of five enzymes that permit the conversion of the enantiomeric pair of mandelates to benzoate; oxidative catabolism of benzoate on to succinate and acetyl coenzyme A is promoted by the enzymes in the closely affiliated β-ketoadipate pathway.[25] Together, these two pathways permit these soil bacteria to use either (*R*)- or (*S*)-mandelate as a sole source of carbon and energy. The other enzymes in the mandelate pathway include an *S*-specific mandelate dehydrogenase, benzoylformate decarboxylase, and two distinct benzaldehyde dehydrogenases. The genes encoding the enzymes in the mandelate pathway were early on considered to constitute an operon/regulon that is inducible by either enantiomer of mandelic acid[26] and, moreover, can be cotransduced;[27] *in vivo* cross-linking experiments have led to the notion that these enzymes may be closely associated with each other inside the bacterial cells.[28]

The gene for MR from *P. putida* was cloned into *Pseudomonas aeruginosa* and expressed in a collaboration between the Gerlt and Kenyon laboratories.[29] Selection of the cloned gene was based upon the ability of the *P. aeruginosa* to grow on (*R*)-mandelate as the sole carbon source by virtue of the absence of MR in its mandelate pathway. Subcloning and sequence analysis of the DNA containing the MR gene permitted the amino acid sequence to be deduced and the exact molecular weight of the enzyme's subunit to be determined (M_r 38 570).

The plasmid containing the subcloned gene (pSCR1) did not permit very generous levels of MR expression, however, and so a novel strategy using α-phenylglycidate was devised to create an overproducing expression system.[30] This overproducing strain has been used to generate generous quantities of enzyme (up to 40 mg of highly purified enzyme per liter of cultured cells).

Recent Mechanistic Studies

Since the early work had strongly implicated carbanionic character at the carbon α to the carboxylate of mandelate in the MR-catalyzed reaction, the Kozarich and Kenyon groups in 1988 launched a collaborative effort to see if more direct evidence could be found for a carbanionic intermediate.[31] Accordingly, *p*-(bromomethyl)mandelate (**1**) (Scheme 3) was synthesized and its reaction with mandelate racemase was evaluated. The reaction of **1** was observed both by UV spectroscopy and by the use of a bromide ion electrode to follow bromide ion release quantitatively. The results using the bromide ion electrode are shown in Figure 1. Over and above the nonenzymatic solvolysis of **1**, there is clearly an enzyme-catalyzed release of bromide ion as well. A new product (**4**) with a λ_{max} at 264 nm was formed in addition to the solvolysis

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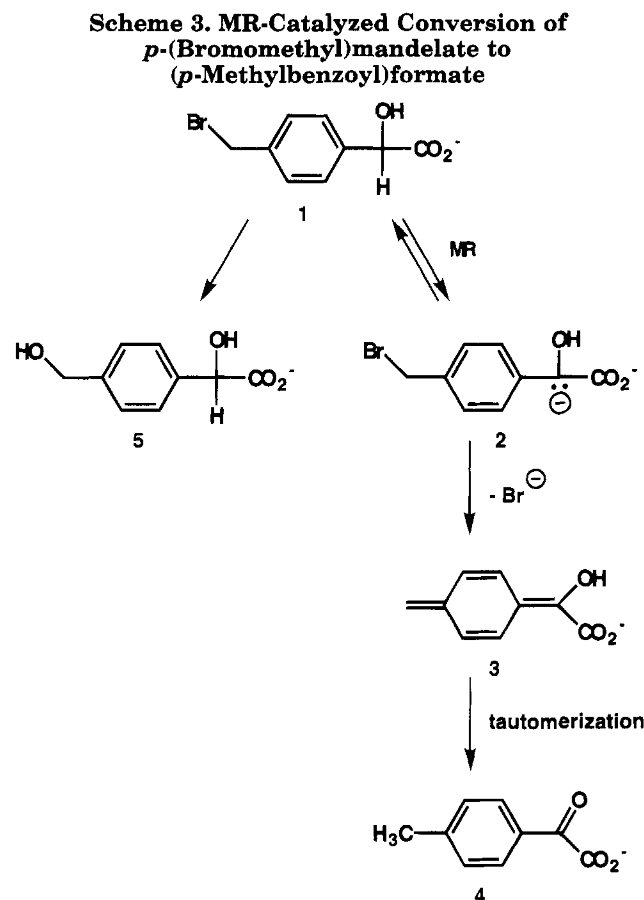
Scheme 3. MR-Catalyzed Conversion of *p*-(Bromomethyl)mandelate to (*p*-Methylbenzoyl)formate



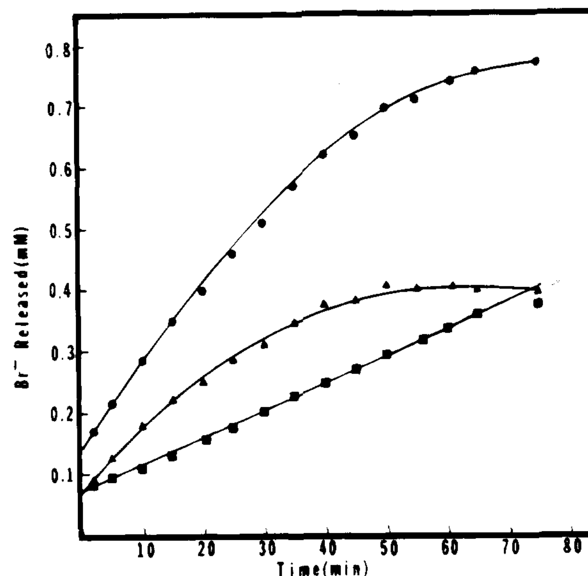


Figure 1. Analysis of bromide ion release from *p*-(bromomethyl)mandelate (1 mM) in 0.1 M MES (pH 6.0) by mandelate racemase: no racemase (■); racemase added (255 units; ●); difference (▲). Reprinted with permission from ref 31. Copyright 1988 American Chemical Society.

product **5** (Scheme 3). In fact, in the presence of a large amount of enzyme, *only* product **4**, identified as (*p*-methylbenzoyl)formic acid, forms. A logical route to **4** is via α-carboxy-α-hydroxy-*p*-xylylene (**3**), which would be expected to tautomerize spontaneously to form **4**; and **3** would most logically come from **2**. These results are fully consistent with the trapping (or diversion) of an intermediate with considerable carbanionic character.

The Kozarich and Kenyon groups also collaborated on a related study wherein propargylglycolate (a mandelate analogue in which the phenyl group of mandelate has been replaced by an acetylenic group) has been shown to be a mechanism-based inactivator of MR, again presumably via an intermediate with carbanionic character, which in this case rearranges to a reactive allenic moiety.[32]

Subsequently, the Kenyon, Kozarich, and Gerlt groups collaborated on a detailed study of MR-catalyzed deuterium/hydrogen exchange at the α-carbon position.[33] These studies used high-resolution gas chromatography/mass spectrometry to examine the various exchange possibilities [e.g., (*R*)-[α-^{1}H]mandelate in D_2O; (*S*)-[α-^{1}H]mandelate in D_2O; (*R*)[α-^{2}H]-mandelate in H_2O; (*S*)-[α-^{2}H]mandelate in H_2O]. In all cases that were examined, exchange reactions were limited to the initial 5−7% turnover so that back exchange from product could be essentially ignored. The results were surprising. Substantially different results were seen in the $R \rightarrow S$ and $S \rightarrow R$ directions. In the $R \rightarrow S$ direction almost no exchange (≤0.4%) of the α-hydrogen in the remaining *R* substrate pool occurred whereas under the same conditions in the $S \rightarrow R$ direction ~5% exchange occurred in the remaining substrate (both after 5−7% turnover). A similar asymmetry in the processing of (*R*)- and (*S*)-[α-^{1}H]-mandelates in D_2O was seen using overshoot experiments (Figure 2).[33-35]

These exchange and overshoot experiments are both consistent with the hypothesis illustrated in Scheme 4. (*S*)-Mandelate has its α-hydrogen removed by a *polyprotic* base (e.g., the ϵ-amino group of a lysine residue) whereas (*R*)-mandelate has its α-hydrogen removed by a *monoprotic* base (e.g., the imidazole nitrogen of a histidine residue). These predictions were borne out by X-ray crystallographic studies described below.

X-ray Crystallography: A Major Surprise

The crystal structure of MR was solved by the Petsko group in collaboration with the Gerlt and Kenyon groups (Figure 3). The original structure was refined to 2.5 Å resolution with an *R*-factor of 18.3%.[8] MR is composed of two major structural domains (an N-terminal α + β domain and a central parallel α/β-barrel) and a third, smaller, irregular C-terminal domain. Thus, MR joins the ever growing group of enzymes whose core α/β-barrel structures resemble closely that of triose-phosphate isomerase (TIM).[36,37] The big surprise was that the overall polypeptide fold was strikingly similar to that of muconate lactonizing enzyme (MLE), whose structure had earlier been solved by Goldman, Ollis, and Steitz.[38] MLE occurs in the β-ketoadipate pathway in the same class of

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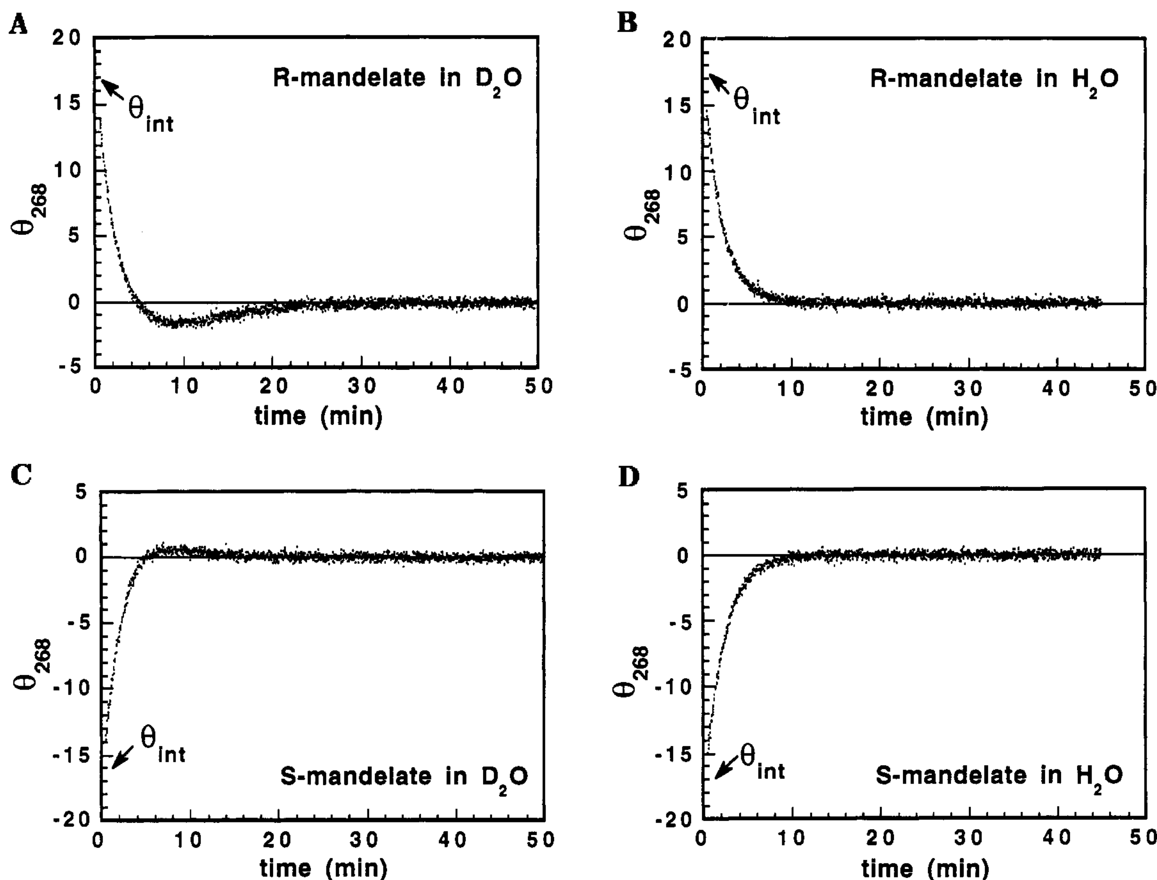


Figure 2. Results of the overshoot experiments. The MR-catalyzed racemization of (*R*)- or (*S*)-[α-^{1}H]mandelate in D_2O (or H_2O as a control) was followed using a circular dichroic assay: (A) racemization of (*R*)-[α-^{1}H]mandelate in D_2O; (B) racemization of (*R*)-[α-^{1}H]mandelate in H_2O; (C) racemization of (*S*)-[α-^{1}H]mandelate in D_2O; (D) racemization of (*S*)-[α-^{1}H]mandelate in H_2O. The overshoot panel in C ($S \rightarrow R$) is attenuated relative to that shown in panel A ($R \rightarrow S$) since, owing to greater deuterium exchange, 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. Reprinted with permission from ref 33. Copyright 1991 American Chemical Society.

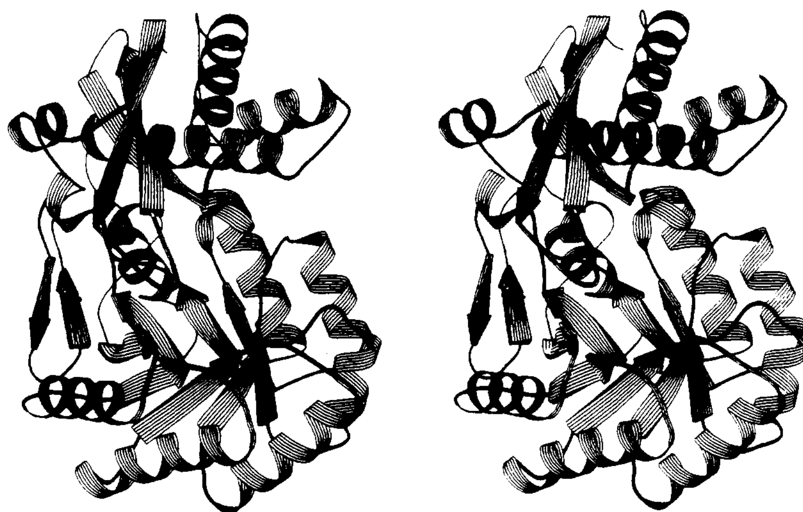


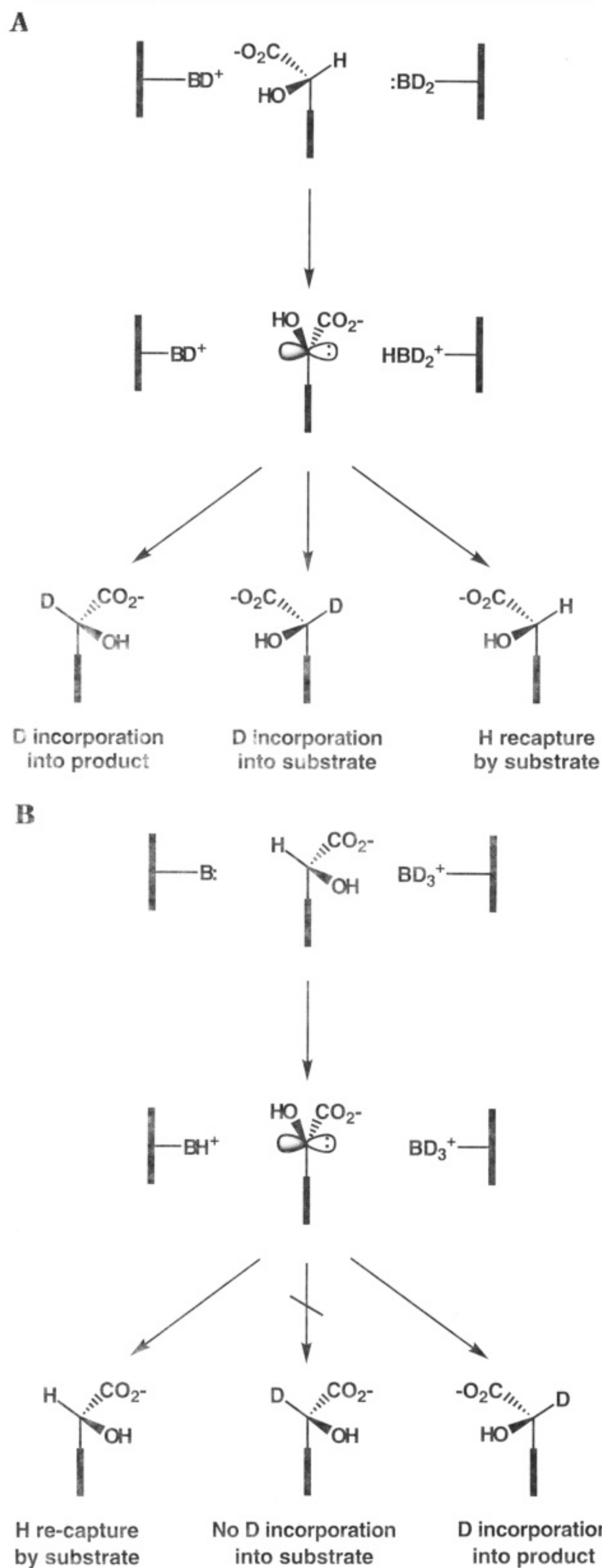
Figure 3. Stereoview of a ribbon diagram showing the three-dimensional structure of the MR backbone. Reprinted with permission from ref 8. Copyright 1991 American Chemical Society.

pseudomonads where MR is found and, ironically, at that time was the only other enzyme involved in mandelate catabolism whose crystal structure had been solved.[6]

MLE catalyzes a Mn^{2+}-dependent addition–elimination reaction in which *cis,cis*-muconate reversibly forms muconolactone (Scheme 5). Although the MR- and MLE-catalyzed reactions appear at first inspection to be very different, the two reactions actually share a common, fundamental step; in the reverse reaction shown in Scheme 5, the α-proton of muconolactone presumably is abstracted by an active site base to generate an intermediate (with carbanionic character), in analogy to the formation of an intermediate in the MR reaction.

The MR/MLE similarities also extend to the secondary, tertiary, and quaternary structures (both enzymes are octamers of 422 symmetry), to the overall subunit

Scheme 4. MR-Catalyzed Fates of the α-Protons in D_2O of (A) (*S*)-Mandelate and (B) (*R*)-Mandelate



packing arrangements (cubic), and to the primary amino acid sequences.[8,29,39] The two enzymes exhibit 25.6% sequence identity. Actually, the residues at the active sites show even greater similarities. MR has

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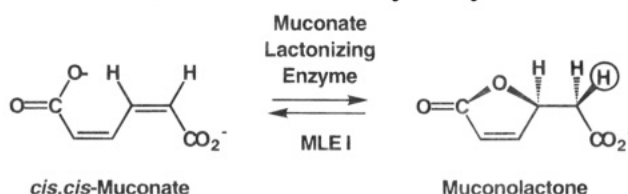
Scheme 5. Reaction Catalyzed by MLE[a]



[a] The encircled proton is the one that is solvent-derived.

its essential Mg^{2+} liganded to Asp195, Glu221, and Glu227. MLE has its essential Mn^{2+} coordinated to Asp198, Glu224, and Glu249, which is spatially equivalent to Glu227 in MR. Also, each has a pair of lysine residues in its active sites which project their ϵ-ammonium/amino groups toward each other in space (Lys164 and Lys166 in MR and Lys167 and Lys169 in MLE).[7] This pair of lysine residues (separated by only one intervening amino acid residue) in the active site regions of MR and MLE is reminiscent of the mechanistically important Lys115–Lys116 pair that was found in the active site of acetoacetate decarboxylase from *Clostridium acetobutylicum* by Westheimer and co-workers.[40–43]

The stereochemistry of the MLE-catalyzed reaction (*syn* addition of the carboxylate group with the *pro-R* proton of the muconolactone product derived from solvent) is consistent with the ϵ-ammonium group of Lys169 being the essential general acidic catalyst in the active site of MLE. By analogy, the ϵ-ammonium/amino group of Lys166 would be expected to be an acid/base catalyst in the active site of MR. These similarities led to the hypothesis that MR and MLE share a common ancestor and that they evolved by the recruitment of needed chemistry followed by refinement of substrate specificity.[44]

In an early X-ray crystallographic experiment *p*-iodomandelate, a weakly active substrate for MR, was diffused into the crystal lattice and its electron density located. The hydrophobic pocket for the phenyl group was readily located. Not only was Lys166 hovering nearby as expected, but His297 also was found to be in close proximity, on the opposite face of the substrate.[8] At this stage, we therefore postulated that Lys166 and His297 were the two active site bases responsible for abstracting the (*S*)- and (*R*)-α-protons, respectively, of the mandelate enantiomers. This hypothesis is consistent with the results of the deuterium exchange experiments described above (Scheme 4) wherein one of the two bases was proposed to be polyprotic and the other monoprotic[33] and provides a framework for an explanation for the "pseudosymmetry" in the active site of MR discussed in the Introduction.

Site-Directed Mutagenesis Experiments

A logical next step was to use site-directed mutagenesis to change some of these key postulated

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active site residues into other, less reactive residues and to examine the consequences. The first residue changed was His297. In a relatively innocuous perturbation it was replaced by an asparagine residue (bearing a carboxamide functionality), which should be able to function neither as an active site base nor as a conjugate acid.

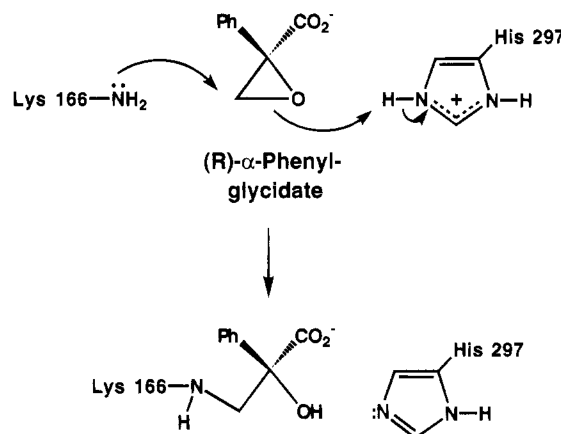
The results with the H297N (asparagine) mutant were striking, definitive, and very informative. (An X-ray crystal structure of the H297N mutant showed no substantial changes in the structure save for the altered residue.) First of all, the H297N mutant was completely devoid of detectable mandelate racemase activity (<0.001%). On the other hand, it was almost fully active (relative to wild-type MR) in its ability to catalyze deuterium exchange in D_2O of the α-proton of (*S*)- but not (*R*)-mandelate. Similarly, H297N was able to catalyze the elimination of bromide ion from (*S*)- but not (*R*)-*p*-(bromomethyl)mandelate, again at a rate quite comparable to that catalyzed by wild-type MR. These results are consistent with the formation of an *intermediate* in the MR-catalyzed reaction pathway. The H297N mutant is "crippled" and cannot deliver a proton to the *R*-face of the intermediate in order to generate (*R*)-mandelate from (*S*)-[α-^{1}H]mandelate. Nevertheless, in D_2O it can catalyze what amounts to a half-reaction in which the intermediate can regenerate (*S*)-mandelate which has acquired a deuterium on the α-carbon.

When Lys166 was changed to an arginine residue, racemization activity fell by a factor of about 1000.[14] Once again, the X-ray crystal structure of the mutant showed only minimal structural perturbations. The K166R mutant was, however, still able to catalyze the elimination of bromide ion from *p*-(bromomethyl)-mandelate at a rate comparable to that of wild-type MR. This time, however, the mutant was specific for (*R*)- and not (*S*)-*p*-(bromomethyl)mandelate. These results are fully consistent with our hypothesis that Lys166 is the *S*-specific base whereas His297 is the *R*-specific base.

Affinity Labeling of Lys166 by α-Phenylglycidate

Even though Fee and Kenyon[23] showed in 1974 that α-phenylglycidate (α-PGA) (Scheme 2) behaved like a classical affinity label for MR, it was only recently that the residue that it alkylates was identified. This was done by first inhibiting the enzyme with (*R*)-α-PGA [(*R*)-α-PGA corresponds in absolute configuration to (*S*)-mandelate; see Scheme 2] at pH 7 and then growing crystals and solving the structure by difference Fourier analysis at 2.0 Å resolution.[12] The results were both rather surprising and pleasing. (*R*)-α-PGA cleanly forms a covalent adduct with the ϵ-amino group of Lys166 by having the NH_2 group attack the distal carbon of the epoxide ring (Scheme 6; Figure 4). Interestingly, an X-ray structure of the competitive inhibitor (*S*)-atrolactate (a nonracemizable homolog of (*S*)-mandelate in which the α-proton has been replaced by a methyl group) with MR reveals that the (*S*)-atrolactate atoms are almost superimposable on the structure for (*R*)-α-PGA-inactivated MR (Figure 4). In the (*S*)-atrolactate–MR structure the α-methyl group [surrogate for the α-proton of (*S*)-mandelate] is pointing directly at Lys166! [In unpub-

Scheme 6. Formation of a Covalent Adduct between Lys166 of MR and (*R*)-α-Phenylglycidate[a]



[a] See also Figure 4.

lished work, Clifton and Petsko have also now shown that the *R* enantiomer of atrolactate, as expected, binds to MR in such a fashion that its α-methyl group points to His297; also, it was found that Glu317 projects its carboxylate group close to the carboxylate of the (*S*)-atrolactate (Clifton and Petsko, unpublished results)]. (*S*)-α-PGA evidently does not form a covalent adduct with MR and appears to bind noncovalently to the active site with a much lower affinity than that of (*R*)-α-PGA.

The fact that Lys166 reacts so rapidly and selectively with (*R*)-α-PGA at pH 7 is consistent with our hypothesis that Lys166 indeed has an altered pK_a (~6) and is the *S*-specific base in the MR-catalyzed reaction. Finally, the structure of (*S*)-atrolactate with MR at high resolution (2.0 Å) revealed for the first time the probable interactions of the substrate mandelate with the essential Mg^{2+} and active site functional groups. This led to the hypothesis that mandelate forms a *bidentate chelate* with MR through both its carboxylate group and the α-hydroxy group. Moreover, the carboxylate group of the bound mandelate is sufficiently close to Glu317 (~2.7 Å) to share a hydrogen with its carboxylate.

Because the K166R mutant is so relatively inactive in abstracting the α-proton from (*S*)-mandelate, (*S*)-mandelate itself could be cocrystallized with it.[14] The results of the crystal structure determination of the enzyme–substrate complex were quite gratifying; (*S*)-mandelate was bound to K166R exactly in the manner in which (*S*)-atrolactate was bound to wild-type MR.

Possible Roles for Glu317 and Asp270

As noted above, the carboxylate group of Glu317 is within hydrogen-bonding distance of the carboxylate groups of both (*R*)- and (*S*)-atrolactates when they are bound to MR. Since the atrolactates are potent competitive inhibitors with respect to the corresponding enantiomers of mandelate, it is a reasonable hypothesis that Glu317 serves as a general acid catalyst for the MR-catalyzed racemizations of both (*R*)- and (*S*)-mandelate by helping to stabilize an intermediate in the reaction pathway. In order to test this postulated role for Glu317, it was recently mutated to a glutamine to generate the E317Q mutant.[13] Since glutamine is sterically extremely similar to glutamate, one would expect only a minimal perturba-

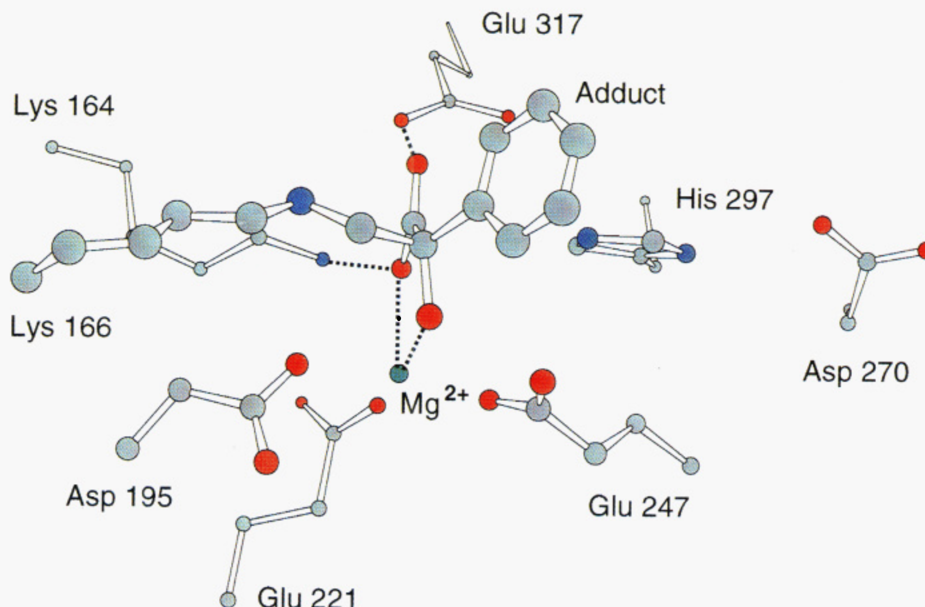


Figure 4. X-ray structure of the active site of MR inactivated by (R)-α-phenylglycidate. The epoxide ring of the inhibitor has alkylated the ε-amino group of Lys166. Both (S)-atrolactate and (S)-mandelate bind in a very similar (but noncovalent) fashion (see text).

tion on the MR structure as was shown experimentally. The k_{cat} values of E317Q were reduced dramatically, however [a (4.5×10^3)-fold reduction for (R)-mandelate as substrate and a 2.9×10^4-fold reduction for (S)-mandelate as substrate]. These results confirm the importance of Glu317 as a putative general acid catalyst in the MR reaction pathway.

Similar results have been found for Asp270, which in the MR structure has its carboxylate group within hydrogen-bonding distance of the imidazole group of His297, one of the two postulated crucial active site bases. Asp270 was mutated to an asparagine residue, which once again represents a minimal structural perturbation as borne out by a high-resolution X-ray structural analysis.[45] As with E317Q, the D270N mutant has a dramatically reduced k_{cat} value for racemization ($\sim 10^3$ reduction). In this case, however, D270N *can* catalyze exchange of the α-proton of (S)-mandelate [but not (R)-mandelate] in D_2O. In other words, D270N behaves like H297N. It appears then that Asp270 and His297 function as a "couplet" or "dyad" of amino acid residues in serving as a general base in the MR reaction. This is reminiscent, of course, of the Asp-His dyad that has appeared regularly in serine proteases.[46]

Catalytic Mechanism

The proposed minimalist catalytic mechanism for the MR-catalyzed reaction is shown in Scheme 7. Only the key amino acid side chain residues are shown for simplicity.

We propose that, during catalysis, Glu317, through hydrogen bonding, partially protonates the second carboxylate oxygen of mandelate, thus significantly increasing the acidity of the α-proton of mandelate.[15] Chelation of the mandelate by the divalent metal ion also has the same effect.[47,48] Finally, the hydrogen bond of mandelate's carboxylate group to the positively charged ε-ammonium group of Lys164 should also contribute to the electrostatic stabilization of the intermediate. Thus, we propose that MR catalyzes facile abstraction of the α-protons of the mandelates by a concerted general acid (using Glu317 in each case) and general base [His297 for (R)- and Lys166 for (S)-mandelate] mechanism that leads to formation of an enolic intermediate (*vide infra*). By microscopic reversibility, the "ketonization" of the intermediate will also be general acid, general base catalyzed, with the carboxylate group of Glu317 serving as the general basic catalyst and the conjugate acid of either Lys166 or His297 serving as the general acidic catalyst.

Accounting for the Rapid Rate: The Gerlt and Gassman Proposal

The conclusion that the mechanism of the MR-catalyzed reaction involves concerted general acid–general base catalysis cannot quantitatively explain the rapid rate of abstraction of the α-proton of the substrate ($pK_a \sim 29$) by the weakly basic functional groups of Lys166 and His297 (the pK_as of their conjugate acids are ~ 6). Without a mechanism to stabilize the intermediate that results from α-proton abstraction, the large ΔpK_a between the substrate and the active site bases means that the expected thermodynamic barrier for formation of the intermediate is too large for it to be kinetically competent. However, the exchange reaction catalyzed by H297N *demands* the presence of an intermediate. This problem of "too unstable" intermediates is not unique to the MR-catalyzed reaction: it is a general problem in mechanistic enzymology that occurs in the reactions catalyzed by several enzymes in primary metabolism, e.g., triose-phosphate isomerase, enolase, citrate synthase, aconitase, and fumarase.

Gerlt and the late Paul G. Gassman proposed a general explanation for the rapid rates of enzyme-

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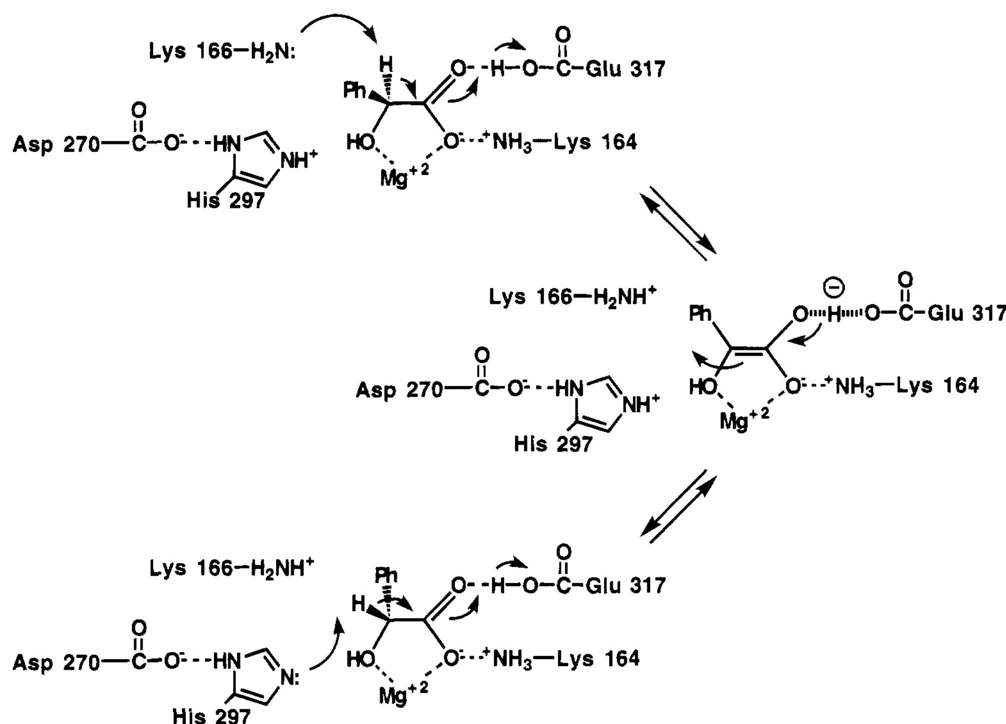
Scheme 7. Postulated Minimal Mechanism of Action of MR[a]



[a] The enzymes' carboxylates (of Asp195, Glu221, and Glu247) that chelate the Mg^{2-} ion are omitted for clarity.

catalyzed α-proton abstraction from weakly acidic carbon acids such as mandelate. A neutral enol would be the intermediate if the proton from an active site general acidic catalyst were transferred to the substrate. However, the pK_a of the enol intermediate would be closely matched to the pK_a of the general acidic (electrophilic) catalyst. For example, in the MR-catalyzed reaction, the pK_as of both the enol (enetriol) intermediate[49] and Glu317 are ~7 (W. Barrett, S. Budihas, and J. A. Gerlt, unpublished observations).

However, Gerlt and Gassman[50,51] proposed that the hydrogen bond involving the proton "shared" by the intermediate and the electrophilic catalyst is a "short, strong" hydrogen bond (designated by ···· in Scheme 7). In this type of hydrogen bond, the proton is physically located *midway* between the intermediate and the electrophilic catalyst rather than being associated with the intermediate one-half the time and with the electrophilic catalyst one-half the time. The importance of this proposal is that "short, strong" hydrogen bonds have strengths that approach 20 kcal/mol in comparison to "normal" hydrogen bonds that have strengths of only a few kilocalories/mole. Such an "enolic" intermediate would be significantly stabilized relative to the substrate, allowing it to be kinetically competent and quantitatively explaining the rapid rates of the α-proton abstraction reactions.

Implications for Other Enzymatic Reactions

The relationships of the MR mechanism with those of other racemases and epimerases have been reviewed by us recently and will not be repeated here.[11] Since that review, papers on glutamate racemase from the Knowles laboratory have appeared that are especially noteworthy as they found several important mechanistic parallels with MR (and, of course, some intriguing differences).[52–54]

Very recently, sequence data bases have been examined and five additional open reading frames (orfs) related to the MR/MLE family have been found.[55] Although the reactions catalyzed by four of these are still uncertain, one of these orfs (f587) has been identified as encoding galactonate dehydratase (Gal D) from *Escherichia coli*. Like mandelate, galactonate is an α-hydroxy acid which also has a proton on the α-carbon. In the course of its catalytic mechanism, Gal D must also promote the abstraction of a proton α to a carboxylate group in order to execute the required elimination of H_2O (Scheme 8).

When one aligns the deduced amino acid sequences of MR and Gal D, Gal D has an Asp-His dyad that is homologous to the Asp-His dyad found in the sequence and the active site of MR, where His297 is the *R*-specific base. However, Gal D lacks a homologue for Lys166 that is the *S*-specific general base in MR. Of course, it does not need a homologue for Lys166 since the absolute configuration of the α-carbon of galactonate is *R*. Thus, it is not a surprise that only an *R*-specific base is present in the sequence of Gal D.

On the basis of these results, we have postulated that MR, MLE, and Gal D all belong to a superfamily of enzymes that has evolved to perform the specific

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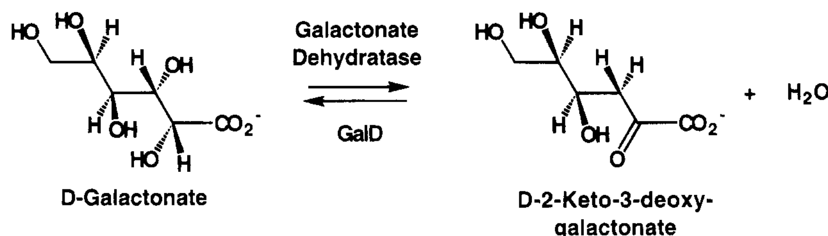
Scheme 8. Reaction Catalyzed by Gal D



task of abstracting protons from the α-carbons of carboxylic acids. In contrast to other enzyme superfamilies whose members differ in substrate specificity but not chemistry (e.g., the serine proteases), the enzymes in the MR/MLE/Gal D superfamily generate and stabilize enolic intermediates by proton abstraction from *either or both* sides of the active site, and the processing of the enolic intermediate to product is determined by the particular active site architecture. A larger perspective here is that understanding the fundamental chemistry behind enzymatic transformations can contribute mightily to the understanding of the evolutionary origins of enzymes.

Conclusions

Mandelate racemase catalyzes the equilibration of (R)- and (S)-enantiomers of mandelate by using general acids and general bases. The pK_a values for the R-specific base (His297) and the S-specific base (Lys166) are about equal (~6). The pK_a of the ϵ-ammonium group of Lys166 is evidently lowered by about 4 pK units by electrostatic effects at the active site. His297 has a more nearly normal pK_a value for a histidine imidazole group. This electrostatic lowering of the pK_a of Lys166 down to close to that for His297 can account for the "pseudosymmetry" in the MR reaction that results in k_{cat} and K_m values for the MR reaction that are very nearly identical in both directions;[3,4,56] pH–rate profiles are also remarkably similar in both directions.[12] The divalent metal ion (e.g., Mg^{2+}) plays a crucial role in chelating the mandelate and helping to lower the acidity of the α-proton that is abstracted.

Finally, the structure–function relationships among mandelate racemase, muconate lactonizing enzyme, and galactonate dehydratase not only indicate that these enzymes evidently have evolved from a common ancestor to perform the necessary (and somewhat daunting) chemical task of abstracting protons α to a carboxylate that have relatively high pK_a values, but also lead to the expectation that other examples of this superfamily will be revealed as new open reading frames appear in the data bases.

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