Mechanism of the Reaction Catalyzed by Mandelate Racemase: Importance of Electrophilic Catalysis by Glutamic Acid 317†

Bharati Mitra,‡ Abraham T. Kallarakal, John W. Kozarich,§ and John A. Gerlt*,||

Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742

James G. Clifton and Gregory A. Petsko*

Rosenstiel Center for Biomedical Sciences, Brandeis University, Waltham, Massachusetts 02254

George L. Kenyon

Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143

Received August 25, 1994; Revised Manuscript Received November 15, 1994⊗

ABSTRACT: In the high-resolution X-ray structure of mandelate racemase (MR) with the competitive inhibitor (*S*)-atrolactate bound in the active site [Landro, J. A., Gerlt, J. A., Kozarich, J. W., Koo, C. W., Shah, V. J., Kenyon, G. L., Neidhart, D. J., Fujita, J., & Petsko, G. A. (1994) *Biochemistry 33*, 635−643], the carboxylic acid group of Glu 317 is hydrogen-bonded to the carboxylate group of the bound inhibitor. This geometry suggests that the carboxylic acid functional group of Glu 317 participates as a general acid catalyst in the concerted general acid−general base catalyzed formation of a stabilized enolic tautomer of mandelic acid as a reaction intermediate. To test this hypothesis, the E317Q mutant of MR was constructed and subjected to high-resolution X-ray structural analysis in the presence of (*S*)-atrolactate. No conformational alterations were observed to accompany the E317Q substitution at 2.1 Å resolution. The values for k_{cat} were reduced 4.5 × 10^3-fold for (*R*)-mandelate and 2.9 × 10^4-fold for (*S*)-mandelate; the values for k_{cat}/K_m were reduced 3 × 10^4-fold. The substrate and solvent deuterium isotope effects measured for both wild-type MR and the E317Q mutant are not multiplicative when deuteriated substrate is studied in D_2O, which suggests that the reactions catalyzed by both enzymes are stepwise and involve the formation of stabilized enolic intermediates. In contrast to wild-type MR, E317Q does not catalyze detectable elimination of bromide ion from either enantiomer of *p*-(bromomethyl)mandelate. However, E317Q is irreversibly inactivated by racemic α-phenylglycidate at a rate comparable to that measured for wild-type MR. Taken together, these mechanistic properties confirm the importance of Glu 317 as a general acid catalyst in the reaction catalyzed by wild-type MR. The k_{cat} for wild-type MR and the reduction in k_{cat} observed for E317Q are discussed in terms of the analysis recently described by Gerlt and Gassman for understanding the rates and mechanisms of enzyme-catalyzed proton abstraction reactions from carbon acids [Gerlt, J. A., & Gassman, P. G. (1993) *J. Am. Chem. Soc. 115*, 11552−11568; Gerlt, J. A., & Gassman, P. G. (1993) *Biochemistry 32*, 11943−11952].

Mandelate racemase (MR,[1] EC 5.1.2.2) from *Pseudomonas putida* ATCC 12633 catalyzes the equilibration of the (*R*)- and (*S*)-enantiomers of mandelate (Kenyon & Hegeman, 1979; Gerlt et al., 1992):

The mechanism of the reaction catalyzed by MR involves two proton transfer reactions in the conversion of the substrate carbon acid[2] to the product carbon acid: the α-proton[2] of the substrate enantiomer is abstracted by an active site general base catalyst, and the α-proton of the product enantiomer is derived from solvent *via* an active site general acid catalyst. This mechanism, often termed a "two-base" mechanism, is supported by a variety of mechanistic investigations (Kenyon & Hegeman, 1979; Gerlt et al., 1992). For example, when the reaction is conducted in D_2O with protiated (*R*)- or (*S*)-mandelate as substrate, the α-hydrogen of the product enantiomer is derived quantitatively from the solvent. Importantly, when (*S*)- but not (*R*)-mandelate is used as substrate, solvent deuterium is rapidly incorporated into the remaining pool of substrate (Powers et al., 1991). The simplest mechanism that explains these observations is that two different general acid−general base catalysts

† This is paper 18 in a series on mandelate racemase; paper 17 is Landro et al. (1994). This research was supported by Grant GM-40570 (J.A.G., G.L.K., J.W.K., and G.A.P.) from the National Institutes of Health.

‡ Present address: Department of Biochemistry, Wayne State University School of Medicine, Detroit, MI 48201.

§ Present address: Department of Biochemistry, Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065-0900.

|| Present address: Department of Biochemistry, 600 South Mathews Ave., University of Illinois, Urbana, IL 61801.

⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1995.

[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; LB, Luria broth; MDH, (*S*)-mandelate dehydrogenase; MR, mandelate racemase.

[2] In this paper we use the term "carbon acid" to describe a molecule in which a proton is bonded to a carbon atom adjacent to a carbonyl or carboxylic acid group. This proton is termed the α-proton.

0006-2960/95/0434-2777$09.00/0

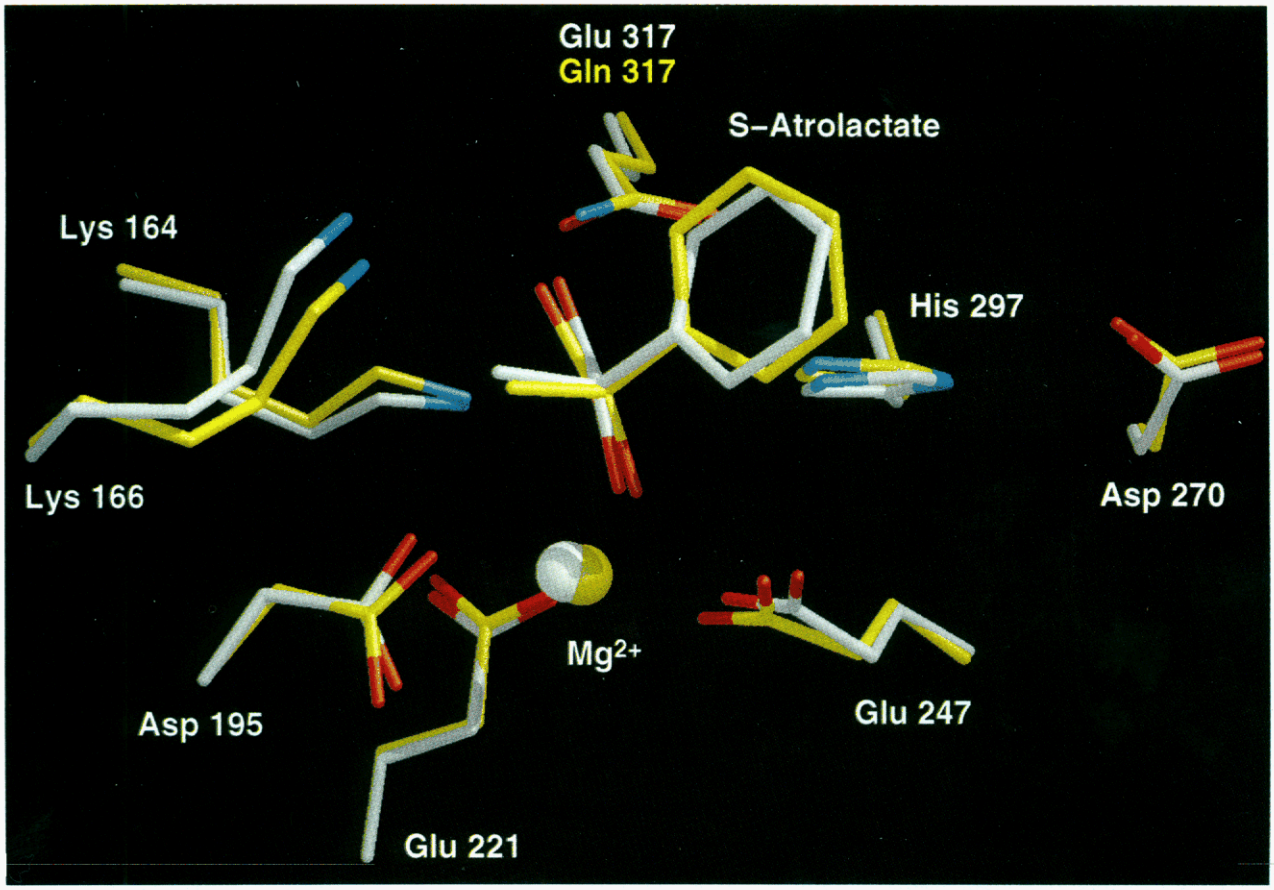

FIGURE 1: Superpositioning of the X-ray structures of the active site of wild-type MR with (*S*)-atrolactate bound in the active site (white; Landro et al., 1994) and of E317Q with (*S*)-atrolactate bound in the active site (yellow). See the text for experimental details.

participate in the proton transfer reactions to and from the α-carbon (a "two-base mechanism").

The high-resolution structure of the competitive inhibitor (*S*)-atrolactate bound in the active site of wild-type MR is shown in Figure 1 (white structure; Landro et al., 1994). The geometry of this complex suggests that the active site general base catalyst that abstracts the α-proton from (*S*)-mandelate is the ϵ-amino group of Lys 166 and that the active site general base catalyst that abstracts the α-proton from (*R*)-mandelate is the imidazole group of His 297 (Neidhart et al., 1991; Landro et al., 1994). The conjugate acids of Lys 166 and His 297 are envisioned to serve as the proton donors in the formation of (*S*)- and (*R*)-mandelates, respectively. That the conjugate acid of Lys 166 is polyprotic while the conjugate acid of His 297 is monoprotic is in accord with the observed exchange of solvent isotope into unreacted (*S*)- but not (*R*)-mandelate (Powers et al., 1991).

These structural and mechanistic data for wild-type MR are equivocal in establishing the timing of the proton transfer reactions to and from the α-carbon. If these proton transfer reactions occurred simultaneously, the conversion of the bound substrate enantiomer to the bound product enantiomer would be concerted. If these reactions occurred sequentially, the conversion of the bound substrate enantiomer to the bound product enantiomer would be stepwise. However, the structural and mechanistic data for the H297N mutant of MR do allow the timing of the proton transfer reactions to be established (Landro et al., 1991). While the H297N mutant of MR does not catalyze any detectable racemization of either enantiomer of mandelate, it does catalyze the facile exchange of the α-proton of (*S*)- but not (*R*)-mandelate with D_2O solvent. This exchange reaction *requires* that a stabilized intermediate be formed as the product of abstraction of the α-proton. Since the rate of the exchange reaction catalyzed by the H297N mutant (~100 s^{-1} in D_2O) is similar to the rate of the racemization reaction catalyzed by wild-type MR (~200 s^{-1} in D_2O, *vide infra*), the implication is that the racemization reaction catalyzed by wild-type MR also occurs *via* the formation of a transiently stable intermediate, i.e., *via* two sequential proton transfer reactions. The formation of a transiently stable, electron-rich intermediate is also indicated by the elimination of halide ions from *p*-(halomethyl)mandelates catalyzed by both wild-type MR (Lin et al., 1988) and the H297N mutant (Landro et al., 1991).

That the reaction catalyzed by wild-type MR involves sequential proton transfer reactions involving a transiently stable intermediate identifies a significant mechanistic problem. The occurrence of an intermediate is intriguing since the pK_a of the α-proton of the substrate greatly exceeds those of the active site general base catalysts (Thibblin & Jencks, 1979). Kresge and his co-workers have measured the pK_a of the α-proton of mandelic acid to be 22.0 (Chiang et al., 1990); the pK_a of the α-proton of mandelate anion has been estimated to be ~29 (Gerlt et al., 1991). From both the observed pH dependence of the rate of exchange of the α-proton of (*S*)-mandelate with solvent catalyzed by H297N and the observed pH dependence of k_{cat} for wild-type MR with either (*R*)- or (*S*)-mandelate as substrate (Landro et al., 1991), the pK_as of the conjugate acids of the general base catalysts Lys 166 and His 297 are thought to be ~6. From transition-state theory and the assumption that the rate of the reaction is limited by the thermodynamics of the transfer

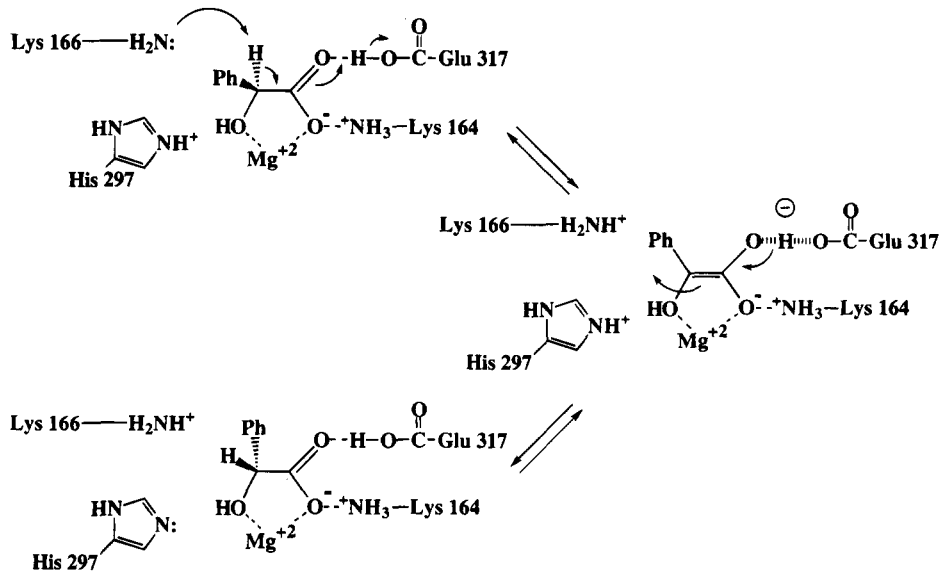

FIGURE 2: Proposed mechanism of the reaction catalyzed by mandelate racemase with concerted general acid–general base catalyzed formation of an enolic intermediate stabilized by a short, strong hydrogen bond (|||||) with Glu 317 (Gerlt & Gassman, 1993a,b). The negative charge (⊖) is dispersed in the hydrogen bond and not localized on the heteroatoms or the bridging proton.

of the α-proton from the substrate to the active site general base catalyst, this difference between the pK_as of the substrate and active site general base catalysts [23 pK_a units if mandelate anion is the substrate or 16 pK_a units if mandelic acid (or its electronic equivalent) is the substrate] is too large to explain the measured k_{cat} for wild-type MR (~500 s^{-1}). Yet, MR does catalyze the rapid equilibration of (*R*)- and (*S*)-mandelates. A similar problem is encountered by other structurally and mechanistically well-characterized enzymes that catalyze reactions which involve transfer of a proton from a weakly acidic carbon acid substrate to a weakly basic active site general base catalyst, including triose-phosphate isomerase, Δ^5-ketosteroid isomerase, and citrate synthase (Gerlt et al., 1991; Gerlt & Gassman, 1993a,b). Thus, if the relationship between the mechanism and the rate of the reaction catalyzed by MR can be understood, the reactions catalyzed by many other enzymes that are able to catalyze the facile abstraction of α-protons of carbon acids might also be better understood.

A proposal to resolve this mechanistic problem for MR was recently put forth by Gerlt and Gassman (1993a,b). The rapid rates of the proton transfer reactions to and from carbon acids can be understood if an active site electrophilic (general acid) catalyst is properly positioned for concerted transfer of a proton toward the carbonyl/carboxylic acid oxygen of the substrate carbon acid as the α-proton is abstracted by an active site general base catalyst (Figure 2). In this *concerted* general acid–general base catalyzed formation of a stabilized enolic intermediate, the k_{cat} can be maximized if the pK_as of the general acid catalyst and the enol tautomer of the substrate carbon acid are *matched*. This matching of pK_as allows significant stabilization of the enolic intermediate relative to the more weakly basic keto tautomer of the substrate carbon acid by the formation of a short, strong hydrogen bond in the solvent-excluded environment of the active site. By this mechanism, the thermodynamic barrier, $\Delta G°$, for formation of the enolic intermediate is reduced in the active site from the value that would be expected for concerted general acid–general base catalysis in solution.[3] A corollary to the stabilization of the enolic intermediate by this mechanism is that the pK_a of the α-proton of the substrate carbon acid is reduced in the acitve site from the value measured in solution.

The positioning of this general acid catalyst adjacent to the carbonyl group of the substrate was also proposed to allow the proton transfer reactions to occur at rates that are controlled by the thermodynamics of the concerted general acid–general base catalyzed formation of the stabilized enolic intermediate. This contrasts with the situation in solution in which a significant intrinsic kinetic barrier, $\Delta G^{\ddagger}_{int}$, makes proton transfer reactions to and from carbon acids slower than expected on the basis of the thermodynamic barrier to the reaction. This effect, together with the reduction in $\Delta G°$ described in the previous paragraph, allows the rates of both the formation of the enolic intermediate and the overall conversion of the substrate carbon acid to the product carbon acid to be understood (Gerlt & Gassman, 1993a,b).

The interactions of the competitive inhibitor (*S*)-atrolactate with the essential Mg^{2+} and functional groups in the active site of wild-type MR are shown in Figure 3 (Landro et al., 1994). This structure reveals that one carboxylate oxygen of the bound inhibitor, and, by analogy, the substrate mandelate, is coordinated to the essential Mg^{2+} and also hydrogen-bonded to the ϵ-ammonium group of Lys 164. Gerlt and Gassman (1992, 1993a,b) proposed that these interactions allow the bound mandelate *anion* to resemble bound mandelic *acid* electronically (Figure 2). This effect reduces the pK_a of the α-proton of the substrate from 29 to 22 (*vide infra*).

The transfer of a proton from an active site general acid catalyst [distinct from the general acid catalyst that protonates the enolic intermediate to form the product enantiomer of mandelate (the conjugate acid of either Lys 166 or His 297)] toward the carboxylic acid group of the bound mandelic acid can further reduce the pK_a of the α-proton of the substrate.

[3] W. W. Cleland and M. M. Kreevoy have also recognized and described the importance of short, strong hydrogen bonds (or "low barrier" hydrogen bond using their nomenclature) in reducing $\Delta G°$ for the formation of unstable intermediates in enzyme-catalyzed reactions (Cleland & Kreevoy, 1994).

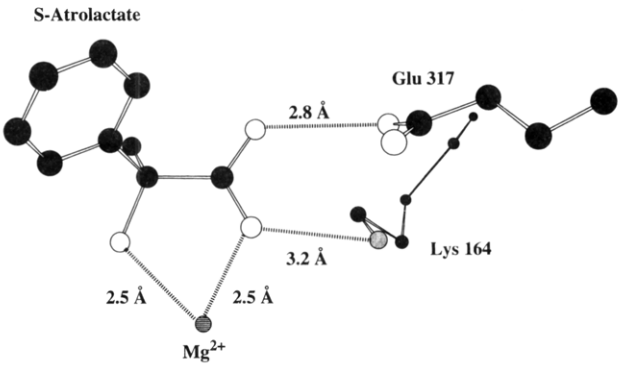

FIGURE 3: Interaction of (*S*)-atrolactate with Mg^{2+} and the functional groups of Lys 164 and Glu 317 in the active site of wild-type MR (Landro et al., 1994).

In the limiting case of complete transfer of a proton from the general acid catalyst to the carboxylic acid group, the pK_a of the α-proton would be reduced by an additional 15 pK_a units to 7 (Gerlt et al., 1991). If the pK_a of the active site general acid catalyst were matched to that of the enol tautomer of mandelic acid so that the proton of the catalyst was equally shared by the catalyst and the enolic intermediate, the pK_a of the α-proton may be reduced by a lesser amount in the transition state for proton transfer, e.g., by ~7.5 pK_a units to ~15 (half that observed for complete transfer; Gerlt & Gassman, 1993a,b). This reduction in the pK_a of the α-proton is sufficient to explain the rate of the MR-catalyzed reaction, assuming that the previously mentioned $\Delta G^{\ddagger}_{int}$ for abstraction of the α-proton is also largely eliminated by the positioning of the general acid catalyst adjacent to the carboxylic acid group in the active site (Landro et al., 1991; Gerlt & Gassman, 1993a,b).

The structural studies of MR reveal the presence of such a general acid catalyst, Glu 317, that is properly positioned for partial proton transfer to the charge-neutralized carboxylate group of the substrate to form a strongly hydrogen-bonded enolic intermediate (Figure 3). The shortest O−O distance between one of the carboxylate oxygens of the bound competitive inhibitor and the carboxylic acid group of Glu 317 is ~2.8 Å, strongly suggesting that Glu 317 is protonated when substrate binds to the active site. The pK_a of the enol tautomer of mandelic acid is ~6 (Chiang et al., 1990); it is reasonable to expect that the pK_a of Glu 317 is also ~6 (Creighton, 1993). Thus, the components and geometry of the active site of MR are appropriate for maximizing the k_{cat} for racemization of mandelate according to the general principles described by Gerlt and Gassman (1993a,b) since the pK_a of the proximal general acid catalyst is likely matched to the pK_a of the enolic intermediate that would be formed from the substrate carbon acid by concerted general acid−general base catalysis (Figure 2).

In this paper we describe the structural and kinetic characterization of the E317Q mutant of MR. This mutant was constructed so that hydrogen bonding between bound substrate and the functional group of residue 317 would be maintained as the enolic intermediate is formed. However, if substantial transfer of a proton from the carboxylic acid group of Glu 317 to the carboxylate group of the charge-neutralized substrate were important to catalysis, this substitution is expected to reduce k_{cat} significantly since the pK_a of a carboxamide group is ~15 (Bordwell, 1988), i.e., ≥9 pK_a units higher than that expected for Glu 317. Thus, in this mutant the pK_as of the general acid catalyst and the enol tautomer of the substrate carbon acid would not be matched, so the enolic intermediate could not be stabilized by a short, strong hydrogen bond. In accord with this expectation, the effect of the structurally conservative E317Q substitution in the active site of MR is to reduce k_{cat} by a factor of ~10^4.

MATERIALS AND METHODS

Materials. Restriction endonucleases, bacteriophage T4 DNA ligase, and bacteriophage T4 polynucleotide kinase were purchased from Boehringer Mannheim. Calf intestine alkaline phosphatase was from Pharmacia LKB. Site-directed mutagenesis was performed by the phosphorothioate method using a kit obtained from Amersham. DNA sequence analysis was performed with the Sequenase kit from U.S. Biochemicals. Synthetic oligonucleotides used for the mutagenesis and DNA sequencing were purchased from the Protein Nucleic Acid Laboratory, University of Maryland, College Park. The techniques described by Sambrook et al. (1989) were used for DNA isolation, for cloning, and for both DNA and protein gel electrophoresis.

(*R,S*)-*p*-(Bromomethyl)mandelate was synthesized following the procedure of Lin et al. (1988). Racemic sodium α-phenylglycidate was prepared by Dr. Vincent M. Powers using the method described by Fee et al. (1974).

Site-Directed Mutagenesis. The template for mutagenesis was M13mp18 into which the 1.9 kb *Eco*RV-*Sst*I fragment of pMRαpg (Tsou et al., 1989) containing the entire gene for MR had been cloned (Landro et al., 1991). The synthetic 18-mer d(CATTGGCTGCAGCGTTTG) was used for constructing the E317Q mutant, where the position of the mismatch is indicated by the underlined base. The entire mutant gene was sequenced by using a series of synthetic primers to verify that no other alterations in nucleotide sequence had occurred.

Preparation of [α-^{2}H]-(R)-Mandelate. [α-^{2}H]-(*R*)-Mandelic acid was prepared by fractional recrystallization of diastereomeric salts of racemic [α-^{2}H]mandelic acid. Racemic [α-^{2}H]mandelic acid was synthesized by using wild-type MR to catalyze the exchange of substrate protium with solvent deuterium. Racemic [α-^{1}H]mandelate (25 g) was incubated in 200 mL of 0.1 M K-HEPES, pH 7.5, and 5 mM $MgCl_2$ in D_2O in the presence of 1.3 mg of wild-type MR. The exchange reaction was monitored by recording the proton NMR spectrum of the reaction mixture. After 2 days, the extent of deuterium incorporation was 64%. The solvent was removed by lyophilization, the solid was redissolved in 200 mL of D_2O, and 2.6 mg more of MR was added. After 10 days, the extent of deuterium incorporation was 95%. The solvent was again removed by lyophilization, the solid was redissolved in 200 mL of D_2O, and once again 2.6 mg of MR was added. After a week, the reaction mixture was acidified with H_2SO_4 to pH 1−2, and the mandelic acid was extracted with 5 volumes of ether. Rotary evaporation of the ether yielded 23 g of mandelic acid. A proton NMR spectrum revealed 98% incorporation of deuterium in the α-position.

The [α-^{2}H]-(*R,S*)-mandelic acid (23 g, 0.15 mol) was dissolved in 50 mL of ethanol with heating. (*R*)-Methylbenzylamine (0.15 mol) was added. The solvent was removed by rotary evaporation. The solid was dissolved in 250 mL of ethanol with heating and left to crystallize at room

temperature. The solid enriched in (*R*)-mandelate was filtered (25 g) and redissolved in 175 mL of ethanol. After three additional recrystallizations, 10 g of the diastereomerically enriched salt was obtained. This salt was dissolved in 2.5 M H_2SO_4, and the mandelic acid was extracted with ether. Rotary evaporation gave 4.8 g of [α-^{2}H]-(*R*)-mandelic acid. The enantiomeric purity of the product was assessed with (*S*)-mandelate dehydrogenase (MDH)[1] and found to be a mixture of 99% [α-^{2}H]-(*R*)-mandelic acid and 1% [α-^{2}H]-(*S*)-mandelic acid.

The [α-^{2}H]-(*S*)-mandelic acid contamination was removed as follows. The nearly completely resolved acid (1 g) was dissolved in 20 mL of 20 mM phosphate buffer, pH 7.0, and 7.5 mg of MDH (Mitra et al., 1993) was added with potassium ferricyanide as the electron acceptor. MDH oxidizes (*S*)-mandelate to benzoylformate, leaving (*R*)-mandelate unaltered. The progress of the reaction was followed by monitoring the decrease in absorbance at 420 nm due to the reduction of the potassium ferricyanide. When the absorbance at 420 nm was constant, the reactino mixture was acidified with H_2SO_4, and the remaining mandelic acid was extracted with ether. Rotary evaporation of the ether yielded 0.75 g of [α-^{2}H]-(*R*)-mandelic acid. The final mandelic acid was 100% enantiomerically pure [(*R*)] and 98% deuteriated.

Purification of Enzymes from Pseudomonas aeruginosa Transformed with pKTtrc/MR. The genes for wild-type MR or E317Q together with the *trc* promoter were cloned into the broad-host range vector pKT230 as previously described (Landro et al., 1991). The plasmid containing the gene for E317Q was designated pKTtrc/E317Q. *P. aeruginosa* (ATCC 15692) was then transformed with either pKTtrc/WT (Landro et al., 1991) or pKTtrc/E317Q and grown in LB media supplemented with 1 mg/mL streptomycin. The purificatin of the proteins was carried out according to established protocols (Ransom et al., 1988). Since E317Q had very low racemase activity, SDS−PAGE instead of enzyme assays was used to monitor column fractions for the E317Q protein.

Crystallization of E317Q. E317Q was cocrystallized with the inhibitor (*S*)-atrolactate by the handing drop method, using slight modifications to the procedure published for wild-type MR (Neidhart et al., 1991). Stock solutions of E317Q were diluted with 50 mM Tris, pH 7.5, containing 10 mM $MgCl_2$ and 50 mM (*S*)-atrolactate in buffer to give a final solution of ~13 mg/mL E317Q and 5 mM atrolactate. A variable amount of precipitant solution was added to 10 μL of the diluted protein solution. The precipitant solution was unbuffered 30% $NH_4(SO_4)_2$/3% (v/v) acetone. The drops were suspended over 1 mL of precipitating solution at room temperature. Crystals appeared after 1−2 days and were fully grown after 1 week. Suitable single crystals were mounted in quartz capillary tubes directly from the hanging drop shortly before data collection.

Data Collection. All data were collected from a single crystal with Cu Kα X-rays using a Siemens multiwire detector mounted on an Elliot GX-6 rotating anode generator, operated with a fine-focus cup at approximately 30 kV and 30 mA. A cold air stream maintained the crystal temperature around 4° C. Data frames were processed using the XDS program (Kabsch, 1988a,b). Two different orientations were collected and merged using the program XSCALE (Kabsch, 1988b). The space group and unit cell parameters were isomorphous to those of wild-type MR (*I*422; 125.5 × 125.5 × 107.3 Å). The merged 2.0 Å data set had an overall merging *R* factor of 13% on intensity. Based on the statistics of the merging *R* factor *vs* resolution and completeness *vs* signal to noise (σ), the final "working" data set contained 20 034 reflections to 2.1 Å resolution that have intensities greater than 1σ (Table 1).

Table 1: Data Collection Statistics from XSCALE

high-resolution limit (Å)	*R* factor (%)			completeness			
	orientation 1	orientation 2	merging	$I > 0\sigma$	$I > 1\sigma$	$I > 2\sigma$	$I > 3\sigma$
6.00	5.7	7.4	6.5	87.9	86.3	85.1	83.6
4.00	8.1	9.3	8.7	93.3	91.2	89.2	87.3
3.00	11.1	13.3	12.2	93.9	90.1	85.4	81.1
2.50	18.1	19.9	19.0	90.5	80.8	70.4	61.7
2.25	23.1	27.8	25.4	88.4	74.1	58.9	46.8
2.10	29.6	34.9	32.3	80.2	62.8	43.3	28.9
2.00	36.4	39.6	38.1	61.3	44.1	23.9	13.2
total			13.0				

Structure Refinement. Initial difference electron density maps calculated using these data and the phases from the refined structure of wild-type MR indicated that there were no gross structural rearrangements; all of the active site residues, including residue 317, and the inhibitor fit cleanly into the electron density. However, the density for residues 18 through 32 [the "flap"; residues that have been found in alternative conformations in other crystals of MR (Clifton et al., unpublished observations)] was poor. Therefore, the initial model for refinement was the wild-type structure lacking the flap, with residue 317 modeled as alanine (to debias the phases) and with neither water molecules nor the inhibitor present.

The program TNT (Tronrud et al., 1987; Tronrud, 1992) was used for least squares refinement of this model *vs* the observed structure amplitudes from the mutant. After refinement against increasingly higher resolution data, new difference electron density maps were calculated. Both the glutamine side chain of residue 317 and the inhibitor could be placed unambiguously in the difference electron density and were added to the model. Some of the residues in the flap could also be placed into density. Repeated refinement, rebuilding, and the inclusion of water molecules improved the density of the flap somewhat. However, attempts to fit the flap as a single conformation failed; the flap existed in two different conformations within this crystal. The program X-PLOR (Brünger, 1988) was then used to refine the coordinates and relative occupancies for a model with two flap structures. A final series of TNT refinement was performed using individual, but correlated, isotropic temperature factors.

The final structure has an *R* factor of 17.1% *vs* all data to 2.1 Å resolution with intensities greater than 1σ (Table 2). The geometry deviations are all below target values: the rms bond length deviation is 0.010 Å and the rms bond angle deviation is 2.2° (Table 3). This structure contains 2799 protein atoms (including 102 atoms for the second conformation of the flap), 12 inhibitor atoms, 1 magnesium atom, and 111 water molecules. The coordinates for this structure have been deposited in the Protein Data Bank at Brookhaven National Laboratories.

Assay of Mandelate Racemase Activity. The racemase activity of E317Q was quantitated routinely using a polari-

Table 2: *R*-Factor Report to 1.85 Å Resolution from TNT

data set	count	rejected by resolution	% completeness
observed	21 764	1730	79
calculated	25 249	0	100

31 691 matches were found (87% complete)

resolution limits: 20.0−1.85 Å

Bragg spacing (Å)	5.66	3.94	3.33	2.98	2.74	2.56	2.42	2.31	2.22	2.14	total
reflections	2423	2343	2292	2175	2043	1924	1869	1824	1687	1454	20034
R factor	0.19	0.13	0.16	0.18	0.18	0.18	0.18	0.18	0.19	0.20	0.171

Table 3: Geometry Report from TNT

class	no.	rms deviation from ideal values
bond length	2888	0.010 Å
bond angle	3902	2.195°
torsion angle[a]	1701	24.29°
trigonal atom nonplanarity	59	0.009 Å
planar groups	422	0.017 Å
bad contacts	8	0.048 Å
thermal parameter correlation	2403	1.664 Å^2

[a] Torsion angles were not constrained during refinement.

metric assay with either (*R*)- or (*S*)-mandelate as substrate. The MDH-coupled assay described by Hegeman (1970) was used to determine the K_m and k_{cat} of both wild-type MR and E317Q for (*R*)-mandelate (either protiated or deuteriated).

A JASCO 370-DIP polarimeter was used for the polarimetric assay. Assays were carried out at 25 °C in a 10-cm cell (2-mL volume). At 25 °C, the specific molar rotation for (*S*)-mandelate, $[\alpha]_{435}$, is +243° (1.52, H_2O). The racemase activity was measured in 0.1 M K-HEPES, pH 7.5, containing 5 mM $MgCl_2$. Since this assay is not sufficiently sensitive at low substrate concentrations, it was used only to determine the values for k_{cat} for both enantiomers of mandelate using three high substrate concentrations.

For the determination of the value of K_m for (*R*)-mandelate and also for the measurement of substrate and solvent deuterium isotope effects, a spectrophotometric assay using MDH as the coupling enzyme was used. The assay mixture contained 0.1 M K-HEPES, pH 7.5, 5 mM $MgCl_2$, 0.05 mM 2,6-dichlorophenolindophenol, 0.2% Triton X-100, and appropriate amounts of MDH (Mitra et al., 1993). The assay follows the decrease in absorbance at 600 nm that occurs as the dye is reduced. The extinction coefficient for DCPIP reduction is 21.6 mM^{-1} cm^{-1} at pH 7.5 (Armstrong, 1964). Using this assay, racemase activity can be measured using only (*R*)-mandelate as substrate. The assay conditions that differ from the polarimetric assay reflect those required for optimal MDH as well as MR activity. Due to the higher sensitivity of this assay, the very low activity of E317Q as well as the K_m for (*R*)-mandelate (for both wild-type MR and E317Q) could be measured reliably.

Elimination of Bromide Ion from (R,S)-p-(Bromomethyl)-mandelate. The elimination of bromide ion from (*R,S*)-*p*-(bromomethyl)mandelate catalyzed by both wild-type MR and E317Q was followed with an Orion Model 811 pH meter equipped with an Orion Model 94-35 bromide ion electrode (Lin et al., 1988; Landro et al., 1991). The reaction was carried out with 8 mg of wild-type MR or 12 mg of E317Q in 100 mM Na-MES, pH 6.0, containing 1 mM $Mg(NO_3)_2$ in a volume of 2 mL. Each reaction was initiated by adding the substituted mandelate to a final concentration of 1 mM. A control experiment was performed without enzyme to obtain the rate of nonenzymatic elimination of bromide ion.

Kinetics of Inhibition by α-Phenylglycidate. The inhibition experiments were carried out according to the protocol described by Fee et al. (1974). Stock solutions of racemic α-phenylglycidate were freshly prepared in 0.1 M K-HEPES, pH 7.5, containing 5 mM $MgCl_2$. The enzyme was incubated with differing concentrations of the inhibitor in the same buffer at room temperature. At known time intervals, aliquots were removed from the incubation mixture and diluted into 1.0 mL of assay buffer. The assays were initiated by the addition of (*R*)-mandelate. A control experiment was carried out analogously with wild-type MR.

RESULTS AND DISCUSSION

The experiments described in this paper were designed to evaluate the proposal that the carboxylic acid functional group of Glu 317 in the active site of MR functions as a general acid catalyst in concerted general acid−general base catalyzed formation of the stabilized enolic tautomer of mandelic acid as an intermediate (Figure 2). In this mechanism, the pK_as of the carboxylic acid group of Glu 317 and the enol tautomer of mandelic acid are approximately matched so that a short, strong hydrogen bond can form and stabilize the intermediate (Gerlt & Gassman, 1993a,b). The effect of stabilization of the enolic intermediate is to lower the pK_a of the α-proton sufficiently such that the rate of its abstraction by the general base catalyst(s) in the active site of MR can be quantitatively explained.

We have evaluated the importance of Glu 317 in catalysis by replacing it with glutamine (E317Q). We expected that this less acidic isostere would prevent significant transfer of a proton toward the carboxylic acid group of the substrate as the α-proton is abstracted, thereby affording less stabilization of the enolic intermediate: the pK_a of the carboxamide group of glutamine is ~15.1 in aqueous solution (the pK_a of acetamide; Bordwell, 1988) as compared to 6.6 measured for the enol tautomer of mandelic acid (Chiang et al., 1990) and ~6 estimated for the γ-carboxylic group of glutamic acid. Thus, we hypothesized that the importance of general acid catalysis by Glu 317 could be assessed by comparing the kinetic properties of the E317Q mutant with those of wild-type MR. We also anticipated that this substitution would not disrupt the active site geometry, since the carboxamide functional group could be expected to retain hydrogen-bonding interactions with other functional groups and ordered water molecules in the active site.

Structural Characterization of E317Q. The E317Q mutant of MR was constructed and purified to apparent homogeneity as described in the Materials and Methods section. The mutant enzyme was crystallized in the presence of (*S*)-

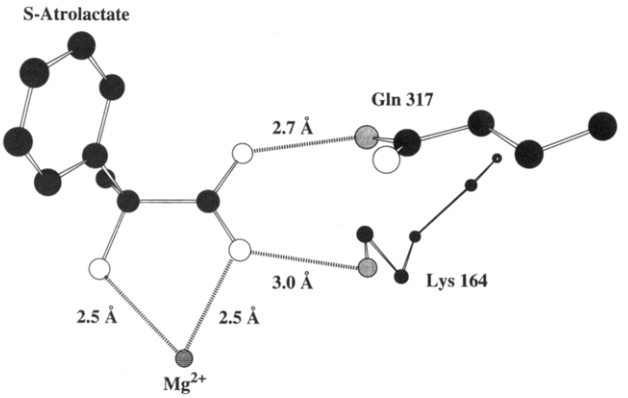

FIGURE 4: Interaction of (*S*)-atrolactate with Mg^{2+} and the functional groups of Lys 164 and Gln 317 in the active site of E317Q.

atrolactate and $(NH_4)_2SO_4$ as described previously for wild-type MR (Landro et al., 1994). The structure was solved to 2.1 Å resolution by difference Fourier analysis relative to the structure of wild-type MR refined to 2.0 Å resolution. The overall structure of the active site of E317Q is shown in Figure 1 (yellow structure), and the details of the interactions of the bound (*S*)-atrolactate with the essential Mg^{2+} and active site functional groups are shown in Figure 4.

A comparison of the active site structures for wild-type MR (Figure 1; white structure) and E317Q (Figure 1; yellow structure) complexed with (*S*)-atrolactate reveals that no detectable differences in active site geometry result from the substitution of a carboxylic acid group by a carboxamide group at residue 317. As shown in Figure 4, (*S*)-atrolactate is coordinated to the essential Mg^{2+} in the active site of E317Q by both its hydroxyl group (2.5 Å) and one of its carboxylate oxygens (2.5 Å); within the estimated error, these distances are identical to those observed for wild-type MR (both 2.5 Å; Figure 3). The carboxylate oxygen of the inhibitor that is coordinated to Mg^{2+} is also hydrogen-bonded to the ϵ-ammonium group of Lys 164 (3.0 Å) in the active site of E317Q (Figure 4); within error, this distance is identical to that observed for the analogous hydrogen bond in the active site of wild-type MR (3.2 Å; Figure 3). Thus, neutralization of the negative charge of the carboxylate group of the bound inhibitor, and, by analogy, substrate, should be unchnaged in the E317Q mutant.

Critical to our mechanistic analysis is the interaction of the carboxylate group of the charge-neutralized inhibitor, and, by analogy, substrate, with the functional group of residue 317 since in wild-type MR the carboxylic acid group of Glu 317 is the putative general acid catalyst. In the active site of E317Q, the other carboxylate oxygen of (*S*)-atrolactate, i.e., the carbonyl group of the charged-neutralized carboxylate group, is hydrogen-bonded to the carboxamide group of Gln 317, as judged by the O−N distance between the bound inhibitor and the enzyme functional group, 2.7 Å (Figure 4). Within error, this distance is identical to that observed for the analogous hydrogen bond involving the carboxylic acid group of Glu 317 in the active site of wild-type MR (2.8 Å; Figure 3).

Thus, the only observable difference in the active site of E317Q relative to wild-type MR is the substitution of the carboxamide functional group for the carboxylic acid functional group at residue 317. Since the E317Q substitution does not detectably perturb the structure of the enzyme beyond the expected functional group interchange, we assume that the mechanistic properties of the E317Q mutant can be used to assess the importance of the carboxylic acid group of Glu 317 in the reaction catalyzed by wild-type MR.

Table 4: Kinetic Constants for Wild-Type MR and E317Q[a]

enzyme	k_{cat} (*R*) → (*S*) (s^{-1})	K_m (*R*) → (*S*) (mM)	k_{cat} (*S*) → (*R*) (s^{-1})
wild type	500 ± 16	0.4 ± 0.05	350 ± 5
E317Q	0.11 ± 0.02	2.4 ± 0.17	0.012 ± 0.001

[a] Assay methods are described in the Materials and Methods section.

Mandelate Racemase Activity of E317Q. The polarimetric assay was used to measure the values for k_{cat} using both (*R*)- and (*S*)-mandelate as substrate at several high concentrations of each enantiomeric mandelate. The values so obtained are compared to those measured for wild-type MR in Table 4. In the (*R*)- to (*S*)-direction, the k_{cat} is reduced by the E317Q substitution by a factor of 4.5×10^3; in the (*S*)- to (*R*)-direction, the k_{cat} is reduced by a factor of 2.9×10^4.

The polarimetric assay cannot be used to measure accurately the values for the K_ms for (*R*)- and (*S*)-mandelates for either wild-type MR or E317Q, since the amount of optical rotation at submillimolar concentrations of mandelate is not sufficient for accurate quantitation. However, when the MR reaction is coupled to the oxidation of (*S*)-mandelate by MDH, the K_m for (*R*)-mandelate can be quantitated accurately. The value so obtained for the E317Q mutant, 2.4 mM (Table 4), exceeds the value measured for wild-type MR (0.4 mM) by a factor of 6. An analogous coupled enzyme assay is not yet available for the racemization of (*S*)-mandelate, so the K_m for (*S*)-mandelate could not be reliably measured. However, from the Haldane relationship, the value for K_m for (*S*)-mandelate can be calculated to be 0.26 mM. This value is indistinguishable from the value, 0.28 mM, that can be calculated analogously for wild-type MR.

From these data we conclude that the substitution of a carboxamide functional group for a carboxylic acid functional group at residue 317 causes a significant reduction in both k_{cat} and k_{cat}/K_m. From the Haldane relationship, the effect of the substitution on k_{cat}/K_m is necessarily the same for both (*R*)- and (*S*)-mandelates, i.e., a 3×10^4 reduction from the value observed for wild-type MR. In contrast, the effect of the substitution on k_{cat} is not the same for both (*R*)- and (*S*)-mandelates. Perhaps the modest differential effect of the mutation on k_{cat} is the result of subtle perturbations in active site structure caused by the substitution. In any event, we conclude that the substitution does, in fact, produce the qualitative effect that is expected if the carboxylic acid group of Glu 317 is a general acid catalyst in the concerted general acid−general base catalyzed formation of the stabilized enol tautomer of mandelic acid (Figure 2).

Substrate and Solvent Isotope Effects. Substrate deuterium isotope effects using [α-^{2}H]-(*R*)-mandelate and solvent deuterium isotope effects were also measured using the coupled enzyme assay. These isotope effects can be used to determine whether proton abstraction from the substrate and proton delivery to yield the product are concerted or occur in two kinetically distinct processes (Hermes et al., 1982; Belasco et al., 1983). The coupled enzyme assay,

Table 5: Substrate and Solvent Deuterium Isotope Effects on k_{cat}[a]

no.	experiment	k_{cat}(wild type) (s^{-1})	k_H/k_D	k_{cat}(E317Q) (s^{-1})	k_H/k_D
1	[α-^{1}H]-(*R*)-mandelate/H_2O	360 ± 6		0.080 ± 0.002	
		400 ± 6		0.052 ± 0.002	
2	[α-^{2}H]-(*R*)-mandelate/H_2O	130 ± 3	2.8	0.023 ± 0.001	3.5
		110 ± 2	3.6	0.023 ± 0.001	2.3
3	[α-^{1}H]-(*R*)-mandelate/D_2O	160 ± 3	2.3	0.026 ± 0.001	3.1
		180 ± 4	2.3	0.014 ± 0.001	3.7
4	[α-^{2}H]-(*R*)-mandelate/D_2O	66 ± 2	5.5 (6.4)[b]	0.018 ± 0.001	4.4 (10.8)[b]
		91 ± 2	4.6 (8.3)[b]	0.0097 ± 0.001	5.4 (8.5)[b]

[a] Values derived from two independent determinations of k_{cat} and K_m. Duplicate assays were performed at substrate concentrations both below and above the values for K_m for each determination of k_{cat} and K_m. [b] The values in parentheses are the products of the substrate (line 2) and solvent (line 3) deuterium isotope effects and are those expected if the mechanism involves concerted proton transfer reactions (Hermes et al., 1982; Belasco et al., 1983).

rather than the less sensitive polarimetric assay, was used to obtain these data.

Four sets of assays were performed for both wild-type MR and E317Q: (1) [α-^{1}H]-(*R*)-mandelate in H_2O, (2) [α-^{2}H]-(*R*)-mandelate in H_2O, (3) [α-^{1}H]-(*R*)-mandelate in D_2O, and (4) [α-^{2}H]-(*R*)-mandelate in D_2O. The values for k_{cat} so obtained are summarized in Table 5.

For both enzymes, the substrate deuterium isotope effect on k_{cat} (compare lines 1 and 2) and solvent deuterium isotope effect[4] on k_{cat} (compare lines 1 and 3) are significantly greater than unity, suggesting that (1) both proton abstraction from the substrate and proton delivery to form the product are each partially rate-determining and (2) substrate binding and product dissociation are faster than the proton transfer reactions.

Importantly, for both enzymes the combined substrate/solvent deuterium isotope effects on k_{cat} (compare lines 1 and 4) are significantly less than the product of the individual substrate and solvent isotope effects. In a concerted reaction in which both proton transfers occur in the same transition state, the combined isotope effect is expected to be the product of the substrate and solvent isotope effects. In a stepwise mechanism in which the proton transfer reaction occurs in distinct transition states, the combined isotope effect is expected to be less than the product, with the precise value being a function of the individual isotope effects and the relative energies of the transition states for the two proton transfer reactions (Hermes et al., 1982; Belasco et al., 1983). That the combined isotope effects are both less than the product of the individual isotope effects suggests that the reactions catalyzed by wild-type MR and E317Q both occur *via* stepwise mechanisms involving a stabilized enolic intermediate.

[4] The observed solvent deuterium isotope effects (line 3 in Table 5) for both wild-type MR and the E317Q mutant are expected to be composites of the isotope effects associated with (1) proton transfer from the conjugate acid of Lys 166 to the α-carbon of the intermediate to generate the (*S*)-mandelate product and (2) proton transfer from and to Glu 317 in the formation and decomposition of the intermediate, respectively. For both enzymes, we expect that the transition states for formation and decomposition of the intermediate will be comparable in energy, thereby masking the effect associated with transfer of a proton from and to Glu 317 in the formation and decomposition of the intermediate. In our studies of the kinetic properties of the K166R mutant of MR (Kallarakal et al., 1995), we observed that, in the (*S*)- to (*R*)-direction in which proton abstraction of the α-proton by Arg 166 and proton transfer from Glu 317 toward the carbonyl oxygen of the substrate are expected to occur in the same rate-limiting transition state, the substrate isotope effect is 3.6 ± 0.2 and the solvent isotope effect is 2.2 ± 0.3. The differing magnitudes of these isotope effects are those expected if the intermediate is stabilized by a short, strong hydrogen bond to Glu 317 (Gerlt & Gassman, 1993a,b).

The conclusion that the reaction catalyzed by wild-type MR occurs *via* a stepwise mechanism is in accord with the observations that (1) deuterium is incorporated into the α-hydrogen of the remaining pool of (*S*)-mandelate when the racemization reaction catalyzed by wild-type MR (Powers et al., 1991) is performed in D_2O solvent and (2) the H297N mutant of MR catalyzes the exchange of the α-hydrogen of (*S*)-mandelate with solvent D_2O at a rate comparable to the rate of the racemization reaction catalyzed by wild-type MR (Landro et al., 1991). At present, we have no independent assessment of the conclusion that the reaction catalyzed by E317Q occurs *via* a stepwise mechanism; such experiments are in progress.

Elimination of Bromide Ion from (R,S)-p-(Bromomethyl)-mandelate. Wild-type MR catalyzes the elimination of bromide ion from both enantiomers of (*R,S*)-*p*-(bromomethyl)mandelate to form *p*-(methyl)benzoylformate at a rate (0.025 s^{-1}) that is ~5 × 10^{-5} the rate of racemization of the enantiomers of unsubstituted mandelate (Lin et al., 1988; A. T. Kallarakal and J. A. Gerlt, unpublished observations). The H297N mutant of MR catalyzes the stereospecific elimination of bromide ion from (*S*)-*p*-(bromomethyl)-mandelate but at a rate (0.012 s^{-1}) that is approximately one-half that observed for wild-type MR (Landro et al., 1991; A. T. Kallarakal and J. A. Gerlt, unpublished observations). The stereospecificity observed in this latter reaction was used as evidence that His 297 is an essential general base catalyst and that in the active site of H297N the ϵ-amino group of Lys 166 retained its ability to act as a general base catalyst.

Lin et al. (1988) proposed that the elimination of bromide ion occurs by partitioning of an intermediate between racemization (*via* protonation of the α-carbon) and elimination. Given the mechanistic proposals put forth by Gerlt and Gassman (1992, 1993a,b), we expect that the common intermediate in both the racemization and elimination reactions catalyzed by wild-type MR and H297N is an enolic intermediate that is stabilized by a short, strong hydrogen bond between the OH group of the enol and the anionic carboxylate group of Glu 317 (or, equivalently, the enolate anion and the carboxylic acid group of Glu 317) since the pK_as of the enol tautomer of mandelic acid and the carboxylic acid group of Glu 317 are likely matched. In the active site of E317Q, the enolic intermediate is not expected to be as stabilized as it is in the active site of wild-type MR (since the pK_as of the enol tautomer of mandelic acid and of the carboxamide group of glutamine are not matched) so its concentration is expected to be less than in the active site of wild-type MR. Thus, we expect that the rate of elimination of bromide ion from (*R,S*)-*p*-(bromomethyl)mandelate cata-

Table 6: Kinetic Parameters for Inhibition by (*R*,*S*)-α-Phenylglycidate

enzyme	k_i (s^{-1})[a]	K_I (mM)[b]
wild type	0.028	0.12
E317Q	0.019	0.69

[a] Rate constant for inactivation. [b] Binding constant.

lyzed by E317Q will be significantly less than that catalyzed by wild-type MR.

The rate of elimination of bromide ion from *p*-(bromomethyl)mandelate cannot be measured with the same precision as the rate of racemization of mandelate, since rapid solvolysis of the benzylic bromide to form *p*-(hydroxymethyl)mandelate ($t_{1/2}$ = 65 min at pH 6.0; Lin et al., 1988) occurs in competition with any enzyme-catalyzed elimination of bromide ion. The observed rate of release of bromide ion from (*R*,*S*)-*p*-(bromomethyl)mandelate in the presence of E317Q is indistinguishable from the rate of the nonenzymatic solvolysis reaction (data not shown). Thus, we conservatively estimate that the rate of elimination of bromide ion from *p*-(bromomethyl)mandelate is at least 25-fold less than that observed in the presence of wild-type MR. We interpret this significantly diminished rate to indicate that the presence of the carboxylic acid group at residue 317 is necessary for formation/stabilization of the enolic intermediate from which elimination of bromide ion can occur.

Inhibition by α-Phenylglycidate. The (*R*)-enantiomer of α-phenylglycidate is an active site-directed, irreversible inactivator of wild-type MR (Fee et al., 1974; Whitman et al., 1985; Landro et al., 1994). The irreversible inactivation occurs *via* alkylation of the ϵ-amino group of Lys 166 (Landro et al., 1994).

The nucleophilic attack of the ϵ-amino group of Lys 166 on the oxirane ring of (*R*)-α-phenylglycidate likely involves general acid catalysis to assist displacement of the alkoxide oxygen. The general acid catalyst is thought to be the imidazolium group of His 297, since the exchange of the α-proton of (*S*)-mandelate with solvent catalyzed by H297N is not inactivated by incubation of the enzyme with α-phenylglycidate (Landro et al., 1994). The carboxylic acid group of Glu 317 is not expected to be involved in the inactivation reaction, except, perhaps, to assist the binding of the carboxylate group of the inactivator in the proper orientation in the active site.

The rates of inactivation, k_i, and the binding constants, K_I, describing the inactivation of both E317Q and wild-type MR by (*R*,*S*)-α-phenylglycidate are compared in Table 6. Although the *KI* for inactivation of E317Q is ~6-fold greater than that measured for wild-type MR, the k_is are similar. (That the k_i for E317Q is 30% less than that measured for wild-type MR may reflect a slightly different binding geometry that is also reflected by the differing values for K_I.) Thus, as expected on the basis of the mechanism of the inactivation reaction, the E317Q substitution should not and does not significantly affect the inactivation by α-phenylglycidate. The inactivation of E317Q by α-phenylglycidate supports our earlier conclusion based upon the structural analysis that the active site geometry is not significantly perturbed by the substitution.

Descriptions of the Reaction Coordinates for Wild-Type MR and E317Q. Qualitatively, the 3×10^4-fold reduction in k_{cat}/K_m and the $0.45-2.9 \times 10^4$-fold reductions in k_{cat} [with the range spanning the reductions observed in the (*R*)- to (*S*)- and (*S*)- to (*R*)-directions, respectively] support our proposal that the carboxylic acid functional group of Glu 317 participates in catalysis as a general acid catalyst in the concerted general acid−general base catalyzed formation of the stabilized enolic tautomer of mandelic acid as an intermediate. Thus, like triose-phosphate isomerase (His 95; Nickbarg et al., 1988) and Δ^5-ketosteroid isomerase (Tyr 14; Xue et al., 1990), the reaction catalyzed by MR is greatly facilitated by an acid functional group that can transfer a proton toward the carbonyl oxygen of the substrate carbon acid as the α-proton is abstracted by the active site general base catalyst (Figure 2).

Are the magnitudes of the reductions in k_{cat} observed for E317Q in *quantitative* accord with the involvement of a general acid catalyst in abstraction of the α-proton from mandelate in the active site of MR? We base the answer to this question on the analysis presented by Gerlt and Gassman (1993a,b) for understanding the rapid rates of enzyme-catalyzed abstraction of the α-protons of carbon acids.

The thermodynamic barrier, $\Delta G°$, for nonenzymatic general acid−general base catalyzed enolization of the mandelic acid using the functional groups present in the active site of MR is expected to be approximately equal to the free energy change associated with the conversion of the keto tautomer of mandelic acid to the enol tautomer of mandelic acid, ~21 kcal/mol, since the pK_as of the general acid catalyst (Glu 317) and the general base catalysts (Lys 166 and His 297) are similar. Although the intrinsic kinetic barrier, $\Delta G^‡_{int}$, has not yet been measured for the enolization of either mandelate anion or mandelic acid in solution, it is likely to be ~12 kcal/mol in analogy to enolization reactions of other carbon acids. From these values and from Marcus formalism (Gerlt & Gassman, 1993a,b) the activation energy barrier, $\Delta G^‡$, for this nonenzymatic reaction is expected to be 24.8 kcal/mol (Figure 5, reaction coordinate a).

From transition-state theory and the k_{cat} for the reaction catalyzed by wild-type MR (~500 s^{-1}), the $\Delta G^‡$ for the concerted general acid−general base catalyzed enolization of bound mandelate is ~14 kcal/mol. This value is shown in Figure 5 by the dotted horizontal line. Thus, the k_{cat} of the catalyzed reaction can be understood if $\Delta G^‡$ for the nonenzymatic reaction can be reduced by 10.8 kcal/mol (a rate acceleration of $\sim 10^8$).

A reduction in $\Delta G°$ can be achieved by the formation of a short, strong hydrogen bond between the enolic intermediate and the carboxylic acid group of Glu 317 since the pK_as of the enol tautomer of mandelic acid and the carboxylic acid group of Glu 317 are approximately matched (Gerlt & Gassman, 1993a,b). Since these pK_as are matched, the proton that is located on the carboxylic acid group of Glu 317 when substrate binds in the active site is expected to be shared equally by the conjugate base of the enol tautomer of mandelic acid and the conjugate base of Glu 317 when the enolic intermediate is formed by abstraction of the α-proton. Although the strengths of short, strong hydrogen bonds can be as large as 20 kcal/mol in the gas phase, in nonaqueous solvents the strengths of these bonds are somewhat less, ≥8 kcal/mol (Gerlt & Gassman, 1993a,b). The formation of the short, strong hydrogen bond is expected to reduce the O−O distance from ~2.8 Å in the enzyme−substrate complex (Figure 3) to ≤2.5 Å in the enzyme−enolic intermediate

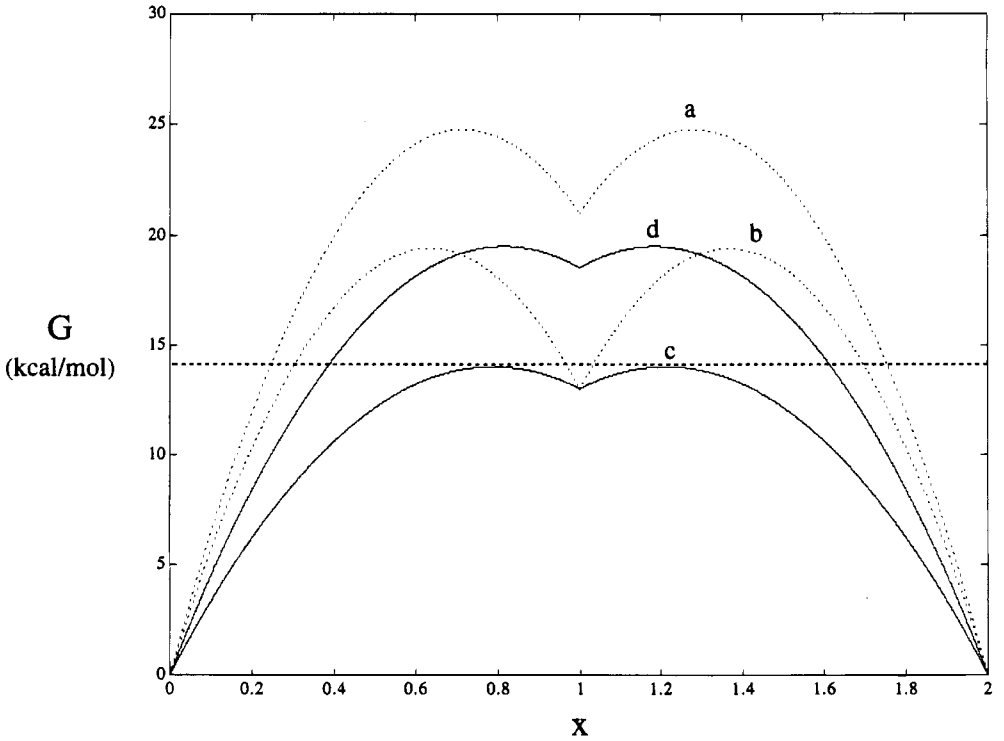

FIGURE 5: Dependence of G on the position of the reaction coordinate, x, for successive enolization and ketonization of mandelic acid. The keto tautomer of the substrate enantiomer of mandelic acid is at $x = 0$, the enol tautomer of mandelic acid (the reaction intermediate) is at $x = 1$, and the keto tautomer of the product enantiomer of mandelic acid is at $x = 2$. Reaction coordinates were generated with eq 1,

$$G = -4\Delta G^{\ddagger}_{int}(x - 0.5)^2 + \Delta G°(x - 0.5) \quad (1)$$

where $\Delta G°$ is the thermodynamic barrier and $\Delta G^{\ddagger}_{int}$ is the intrinsic kinetic barrier for each reaction. The value of G is normalized so that $G = 0$ when $x = 0$. This normalization was accomplished by adding the value of G when $x = 0$ ($\Delta G^{\ddagger}_{int} + \Delta G°/2$) to the value of G calculated with eq 1. The reaction coordinates were calculated assuming (a) $\Delta G° = 21$ kcal/mol and $\Delta G^{\ddagger}_{int} = 12$ kcal/mol, (b) $\Delta G° = 13$ kcal/mol and $\Delta G^{\ddagger}_{int} = 12$ kcal/mol, (c) $\Delta G° = 13$ kcal/mol and $\Delta G^{\ddagger}_{int} = 5.62$ kcal/mol, and (d) $\Delta G° = 18.5$ kcal/mol and $\Delta G^{\ddagger}_{int} = 7.3$ kcal/mol. The reaction coordinates were calculated and plotted with Macintosh MATLAB 3.5, The Mathworks, Inc., Natick, MA.

complex. This small adjustment in the geometry of the active site should be allowed by the flexibility of the side chain of Glu 317.

A reduction in $\Delta G^{\ddagger}_{int}$ can be achieved by the positioning of a proton donor adjacent to the carbonyl oxygen so that the negative charge that develops on the carbonyl oxygen as the α-proton is abstracted by the general base catalyst can be neutralized (Gerlt & Gassman, 1993a,b). Although the precise amount of the reduction in $\Delta G^{\ddagger}_{int}$ is difficult to predict *a priori*, in model reactions significant reductions in $\Delta G^{\ddagger}_{int}$ are achieved by negating the development of a negative charge on the carbonyl oxygen as the α-proton is abstracted. We propose that a reduction in $\Delta G^{\ddagger}_{int}$ is achieved in the active site of MR by the observed positioning of the carboxylic acid functional group of Glu 317 adjacent to the charge-neutralized carboxylate group of bound mandelate (Figures 1 and 3) so that the developing negative charge on the carbonyl oxygen can be neutralized without any entropically unfavorable reorientation of the functional group.

If $\Delta G°$ for formation of the enolic intermediate is reduce by 8 kcal/mol from 21 to 13 kcal/mol by the formation of the short, strong hydrogen bond and $\Delta G^{\ddagger}_{int}$ is unchanged from the value of 12 kcal/mol that characterizes nonenzymatic reactions of carbon acids, $\Delta G^{\ddagger}$ is reduced only to 19.4 kcal/mol (Figure 5, reaction coordinate b). This reduction is not sufficient to explain the observed rate of the reaction catalyzed by wild-type MR.

However, if $\Delta G^{\ddagger}_{int}$ also is reduced from 12 kcal/mol to 5.6 kcal/mol, $\Delta G^{\ddagger}$ is reduced to 14 kcal/mol (Figure 5, reaction coordinate c).[5] This reduction is sufficient to explain the observed rate of the reaction catalyzed by wild-type MR. We note that this combination of reductions in both $\Delta G°$ and $\Delta G^{\ddagger}_{int}$ allows the enolic intermediate (at $x = 1$ on the reaction coordinate) to be transiently stable, as required by the observed exchange of the α-proton of (*S*)-mandelate with solvent catalyzed by H297N (Landro et al., 1991).

Can we explain the reductions in k_{cat} that accompany the E317Q substitution by the same mechanistic analysis? Given the pK_as of the functional groups present in the active site of E317Q (Gln 317, Lys 166, and His 297) and the pK_E for mandelic acid, we expect that a general base-catalyzed mechanism described by $\Delta G° \geq 22.4$ kcal/mol[6] is operative in the active site of E317Q.

The reduction in k_{cat} for E317Q relative to that for wild-type MR corresponds to an increase in $\Delta G^{\ddagger}$ to 19.5 kcal/mol from the value of 14 kcal/mol that describes the reaction catalyzed by wild-type MR. That $\Delta G^{\ddagger}$ is not increased to

[5] The reduction in $\Delta G^{\ddagger}_{int}$ that was used to calculate reaction coordinate c in Figure 5 predicts that the equilibrium constant for formation of the enolic intermediate should be significantly less than unity. This prediction differs from that described by Albery and Knowles (1976) in their analysis of the rates and mechanisms of enzyme-catalyzed reactions (Gerlt & Gassman, 1993a,b).

[6] Calculated from 1.4 kcal/mol × 22 (the pK_a of mandelic acid) − 6 (the pK_a of Lys 166/His 297). If the pK_a of the bound mandelate exceeds that of mandelic acid (the upper limit is 29; Gerlt et al., 1991), the $\Delta G°$ will be greater.

$\geq$25.8 kcal/mol,[7] the value that would be expected for the general base-catalyzed reaction in a nonenzymatic reaction, suggests that both $\Delta G°$ and $\Delta G^{‡}_{int}$ are reduced in the active site of E317Q:

(1) $\Delta G°$ is reduced from the value that describes general base-catalyzed abstraction of the α-proton ($\geq$22.4 kcal/mol). This reduction is possible if the strength of the hydrogen bond between the carboxamide group of Gln 317 and the enolate anion of mandelate is stronger than the hydrogen bond between the carboxamide group of Gln 317 and the carboxylate group of bound mandelate. An increase in the strength of the hydrogen bond to the carboxamide group of Gln 317 is not surprising since strengths of hydrogen bonds increase when the hydrogen bond is charged rather than neutral (Fersht et al., 1985). Thus, the $\Delta G°$ for general base-catalyzed abstraction of the α-proton is reduced but not as much as the $\Delta G°$ for concerted general acid−general base catalysis is reduced in the active site of wild-type MR by the formation of a short, strong hydrogen bond.

(2) $\Delta G^{‡}_{int}$ is reduced from the value that describes nonenzymatic enolization reactions of carbon acids (12 kcal/mol). We attribute the reduction in $\Delta G^{‡}_{int}$ in the active site of E317Q to the positioning of the hydrogen-bonding carboxamide group of Gln 317 adjacent to the carboxylate group of the bound mandelate so that the developing negative charge can be stabilized, although not neutralized, as the α-proton is abstracted by the general base catalyst. This conclusion that the value of $\Delta G^{‡}_{int}$ is reduced in the active site of E317Q suggests that the formation of a short, strong hydrogen bond is not necessary for a reduction in $\Delta G^{‡}_{int}$. However, since values for $\Delta G°$ and $\Delta G^{‡}_{int}$ have not yet been measured, the actual reduction in $\Delta G^{‡}_{int}$ in the active site of either wild-type MR or E317Q is unknown.

To illustrate these proposals, reaction coordinate d in Figure 5 was calculated assuming that $\Delta G° = 18.5$ kcal/mol and $\Delta G^{‡}_{int} = 7.3$ kcal/mol. The $\Delta G^{‡}$ for this profile, 19.5 kcal/mol, exceeds the value observed for the reaction catalyzed by wild-type MR, 14 kcal/mol, by 5.5 kcal/mol, which is in quantitative agreement with the decrease in k_{cat} that accompanies the E317Q substitution. While the values of $\Delta G°$ and $\Delta G^{‡}_{int}$ used in these calculations are not obtained from experimental measurements, we conclude that the rates and mechanisms of the reactions catalyzed by both wild-type MR and E317Q can be described by the mechanistic principles proposed by Gerlt and Gassman (1993a,b).

Conclusions. The carboxylic acid group of Glu 317 in the active site of MR is important for catalysis as revealed by the $0.45-2.9 \times 10^4$-fold decreases in k_{cat} that accompany the E317Q substitution. This reduction in activity supports the proposal that the mechanism of the reaction catalyzed by MR involves concerted general acid−general base catalyzed formation of an enolic intermediate that is stabilized by a short, strong hydrogen bond between the OH group of the enolic intermediate (pK_a 6.6) and the carboxylic acid group of Glu 317 (pK_a likely to be ~6). The k_{cat}s and mechanisms of the reactions catalyzed by both wild-type MR and E317Q can be understood using the analysis recently described by Gerlt and Gassman (1993a,b).

[7] Calculated with Marcus formalism (Gerlt & Gassman, 1993a,b) assuming that $\Delta G° \geq 22.4$ kcal/mol and $\Delta G^{‡}_{int} = 12$ kcal/mol.

REFERENCES

Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5627.
Armstrong, J. M. (1964) *Biochim. Biophys. Acta 86*, 194−197.
Belasco, J. G., Albery, W. A., & Knowles, J. R. (1983) *J. Am. Chem. Soc. 105*, 2475−2477.
Bordwell, F. G. (1988) *Acc. Chem. Res. 21*, 456−463.
Brünger, A. T. (1988) *J. Mol. Biol. 203*, 803−816.
Chiang, Y., Kresge, A. J., Pruszynski, P., Schepp, N. P., & Wirz, J. (1990) *Angew. Chem., Int. Ed. Engl. 29*, 792−794.
Cleland, W. W., & Kreevoy, M. M. (1994) *Science 264*, 1887−1890.
Creighton, T. E. (1993) *Proteins: Structure and Molecular Properties*, p 6, W. H. Freeman, New York.
Fee, J. A., Hegeman, G. D., & Kenyon, G. L. (1974) *Biochemistry 13*, 2533−2538.
Fersht, A. R., Shi, J. P., Knill-Jones, J. W., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature (London) 314*, 235−238.
Gerlt, J. A., & Gassman, P. G. (1992) *J. Am. Chem. Soc. 114*, 5928−5934.
Gerlt, J. A., & Gassman, P. G. (1993a) *J. Am. Chem. Soc. 115*, 11552−11568.
Gerlt, J. A., & Gassman, P. G. (1993b) *Biochemistry 32*, 11943−11952.
Gerlt, J. A., Kozarich, J. W., Kenyon, G. L., & Gassman, P. G. (1991) *J. Am. Chem. Soc. 113*, 9667−9669.
Gerlt, J. A., Kenyon, G. L., Neidhart, D. J., Petsko, G. A., & Powers, V. M. (1992) *Curr. Opin. Struct. Biol. 2*, 736−742.
Hegeman, G. D. (1970) *Methods Enzymol. 17*, 670−674.
Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106−5114.
Kabsch, W. (1988a) *J. Appl. Crystallogr. 21*, 67−71.
Kabsch, W. (1988b) *J. Appl. Crystallogr. 21*, 916−924.
Kallarakal, A. T., Mitra, B., Kozarich, J. W., Gerlt, J. A., Clifton, J. G., Petsko, G. A., & Kenyon, G. L. (1995) *Biochemistry 34*, 2788−2797.
Kenyon, G. L., & Hegeman, G. D. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 50*, 325−360.
Landro, J. A., Kallarakal, A. T., Ransom, S. C., Gerlt, J. A., Kozarich, J. W., Neidhart, D. J., & Kenyon, G. L. (1991) *Biochemistry 30*, 9274−9281.
Landro, J. A., Gerlt, J. A., Kozarich, J. W., Koo, C. W., Shah, V. J., Kenyon, G. L., Neidhart, D. J., Fujita, S., & Petsko, G. A. (1994) *Biochemistry 33*, 635−643.
Lin, D. T., Powers, V. M., Reynolds, L. J., Whitman, C. P., Kozarich, J. W., & Kenyon, G. L. (1988) *J. Am. Chem. Soc. 110*, 323−324.
Mitra, B., Gerlt, J. A., Babbitt, P. C., Koo, C. W., Kenyon, G. L., Joseph, D., & Petsko, G. A. (1993) *Biochemistry 32*, 12959−12967.
Neidhart, D. J., Howell, P. L., Petsko, G. A., Powers, V. M., Li, R., Kenyon, G. L., & Gerlt, J. A. (1991) *Biochemistry 30*, 9264−9273.
Nickbarg, E. B., Davenport, R. C., Petsko, G. A., & Knowles, J. R. (1988) *Biochemistry 27*, 5948−5960.
Powers, V. M., Koo, C. W., Kenyon, G. L., Gerlt, J. A., & Kozarich, J. W. (1991) *Biochemistry 30*, 9255−9263.
Ransom, S. C., Gerlt, J. A., Powers, V. M., & Kenyon, G. L. (1988) *Biochemistry 27*, 540−545.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Thibblin, A., & Jencks, W. P. (1979) *J. Am. Chem. Soc. 101*, 4963.
Tronrud, D. E. (1992) *Acta Crystallogr. A48*, 912−916.
Tronrud, D. E., Ten Eyck, L. F., & Matthews, B. W. (1987) *Acta Crystallogr. A43*, 489−501.
Tsou, A. Y., Ransom, S. C., Gerlt, J. A., Powers, V. M., & Kenyon, G. L. (1989) *Biochemistry 28*, 969−975.
Whitman, C. P., Hegeman, G. C., Cleland, W. W., & Kenyon, G. L. (1985) *Biochemistry 24*, 3936−3942.
Xue, L., Talalay, P., & Mildvan, A. S. (1990) *Biochemistry 29*, 7491−7500.

BI9419902