Articles

Active Site Residues of Glutamate Racemase†

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ABSTRACT: Glutamate racemase, MurI, catalyzes the interconversion of glutamate enantiomers in a cofactor-independent fashion and provides bacteria with a source of D-Glu for use in peptidoglycan biosynthesis. The enzyme uses a "two-base" mechanism involving a deprotonation of the substrate at the α-position to form an anionic intermediate, followed by a reprotonation in the opposite stereochemical sense. In the *Lactobacillus fermenti* enzyme, Cys73 is responsible for the deprotonation of D-glutamate, and Cys184 is responsible for the deprotonation of L-glutamate; however, very little is known about the roles of other active site residues. This work describes the preparation of four mutants in which strictly conserved residues containing ionizable side chains were modified (D10N, D36N, E152Q, and H186N). During the course of this research, the structural analysis of a crystallized glutamate racemase indicated that three of these residues (D10, E152, and H186) are in the active site of the enzyme [Hwang, K. Y., Cho, C.-S., Kim, S. S., Sung, H.-C., Yu, Y. G., and Cho, Y. (1999) *Nat. Struct. Biol. 6*, 422−426]. Two of the mutants, D10N and H186N, displayed a marked decrease in the values of k_{cat}, but not K_M, and are therefore implicated as important catalytic residues. Further analysis of the primary kinetic isotope effects observed with α-deuterated substrates showed that a significant asymmetry was introduced into the free energy profile by these two mutations. This is interpreted as evidence that the mutated residues normally assist the catalytic thiols in acting as bases (D10 with C73 and H186 with C184). An alternate possibility is that the residues may serve to stabilize the carbanionic intermediate in the racemization reaction.

The enzyme glutamate racemase (MurI, EC 5.1.1.3) catalyzes the interconversion of D- and L-glutamic acid and is the source of D-glutamate in most bacterial strains (*1*). D-Glutamic acid is an important biosynthetic building block since it is required in the formation of peptidoglycan that protects bacteria from osmotic lysis (*2*). It is clear that the disruption of peptidoglycan biosynthesis is lethal to bacteria and therefore an understanding of the mechanism of glutamate racemase could be useful in the design of new antibiotics (*3*, *4*).

Glutamate racemase is a member of a small group of amino acid racemases and epimerases (*1*) that operate in a cofactor-independent fashion and include proline racemase (*5*−*8*), aspartate racemase (*9*), and diaminopimelate epimerase (*10*−*14*). These enzymes share a common mechanism that employs two active site cysteine residues in catalysis. In a given reaction direction, one cysteine serves to deprotonate the substrate at the α-position and the other reprotonates the resulting carbanionic intermediate on the opposite face, generating the enantiomeric product (Figure 1). These reactions are of mechanistic interest since it is unusual for enzymes to utilize thiol(ates) as acid/base residues and since these relatively weak bases must abstract a relatively nonacidic proton.

Mechanistic studies on glutamate racemase are consistent with a "two-base" mechanism involving monoprotic acid/base residues. It has been shown that racemization is accompanied by solvent-derived deuterium incorporation into the product enantiomer, but not into recovered starting material, in both reaction directions (*15*, *16*). Furthermore, inactivation with the substrate analogue "aziridino-glutamate" was shown to proceed with covalent labeling of an active site thiol (*17*).

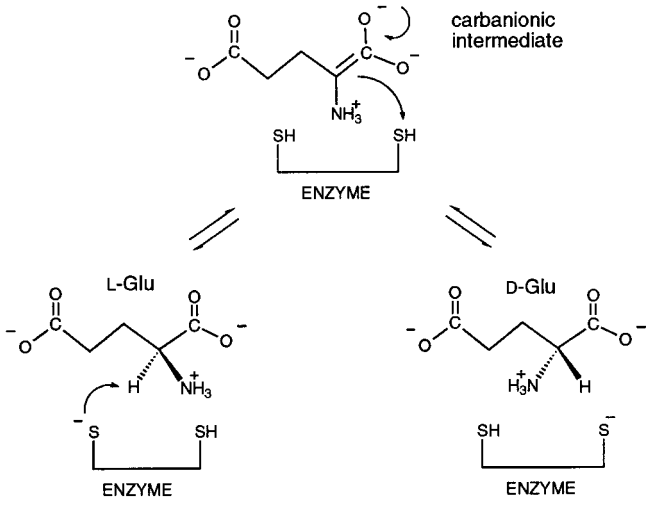

FIGURE 1: Proposed mechanism for the reaction catalyzed by glutamate racemase.

Mutagenesis studies have further supported the notion that two cysteines provide the acid/base residues required for catalysis. With the *Lactobacillus fermenti* enzyme, mutation of either of the cysteine residues to alanine eliminated the

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racemase activity (*18*), and similar results were obtained with Cys-to-Thr/Ala mutants of the *Escherichia coli* enzyme (*19*, *20*). Further studies on the inactive *L. fermenti* mutants indicated that they were each capable of catalyzing the elimination of HCl from opposite enantiomers of *threo*-3-chloroglutamate (*18*). This reaction presumably requires only one catalytic acid/base residue and demonstrates that the active sites were not dramatically perturbed by the mutations. It also suggested that Cys73 is responsible for the deprotonation of D-glutamate and Cys184 is responsible for the deprotonation of L-glutamate.

Recent work on the *L. fermenti* enzyme involved studies on mutants in which either of the active site cysteine residues had been replaced with serine (C73S or C184S) (*21*). It was somewhat surprising to see that these mutants retained significant racemase activity and displayed k_{cat} values within 0.3−3.0% of those observed with the wild-type enzyme. This residual activity allowed, however, for an assessment of the asymmetry introduced into the active site as a result of the mutations. In the wild-type reaction, primary kinetic isotope effects were observed with [2-^{2}H]glutamate in both reaction directions, indicating that both steps of the reaction were partially rate-limiting and the reaction energy profile is somewhat symmetric. The replacement of one of the catalytic cysteine residues with serine resulted in a perturbation of this symmetry such that the deprotonation or reprotonation step involving the alkoxide or alcohol became more cleanly rate-determining. Since the primary kinetic isotope effect measurements using [2-^{2}H]glutamate probe the deprotonation step, it was possible to assign a given base to a given enantiomer of glutamate. In the case of C73S, the V_{max}/K_M isotope effect on D-glutamate increased whereas that on L-glutamate decreased. The opposite trend was observed with C184S, indicating that Cys73 is responsible for the deprotonation of D-glutamate and Cys184 is responsible for the deprotonation of L-glutamate.

Despite a good understanding of the residues directly involved in the deprotonation/reprotonation steps, little is known about other active site residues that are important for catalysis. Toward this end, we performed a sequence alignment of 13 isozymes of glutamate racemase to look for strictly conserved residues that may play a role in catalysis. We focused on Lys, His, Glu, and Asp residues that may participate in either acid/base or electrostatic catalysis (the only conserved cysteines were the two known active site residues). Four such residues were completely conserved in all strains and were Asp10, Asp36, Glu152, and His186 in the *L. fermenti* enzyme. In this paper, we report the effects of conservative mutations on these residues and on the use of primary kinetic isotope effect measurements to probe the extent to which these mutations induce an asymmetry on the reaction profile. During the course of this research, the structure of glutamate racemase from *Aquifex pyrophilus* was solved by X-ray crystallography and strongly indicates that Asp10, Glu152, and His186 are located in the active site of the *L. fermenti* enzyme (*22*).

EXPERIMENTAL PROCEDURES

Materials. Ultrapure potassium phosphate and ultrapure potassium hydroxide were purchased from Aldrich. D- and L-[2-^{2}H]Glutamate were prepared by the method of Tanner et al. (*18*) (Note: an error was found in the Experimental Section of this reference; 6 M NaOH is used in the acylation, not 6 M HCl). The following oligonucleotides were synthesized on a PE Applied Biosystems model 380B DNA synthesizer: (1) CGACGAGCGTGACACCACGATGCC, (2) GCAGAGCGAGGTATGTAGGCGGTGC, (3) GGAGTGATGAATTCTGGCTTGG, (4) CCAAGCCAGAATTCATCACTCC, (5) CTTCGTGGGCAACCAAGGTCAC, (6) GTGACCTTGGTTGCCCACGAAG,(7)CGAAATCGTTCAGCACGGCC, (8) GGCCGTGCTGAACGATTTCG, (9) GGGCTGCACCAACTTCCCG, (10) CGGGAAGTTGGTGCAGCCC. Primers 1 and 2 are complementary to the pUC18 vector. Primers 3 and 4 encode for the D10N mutation, primers 5 and 6 encode for the D36N mutation, primers 7 and 8 encode for the E152Q mutation. and primers 9 and 10 encode for the H186N mutation. Underlined regions encode the amino acid substitutions.

Sequence Alignment. Sequence alignments were performed using the CLUSTAL W 1.81 algorithm courtesy of EMBL-European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw).

Plasmid Construction and Mutant Purification. Plasmids coding for the mutant enzymes were prepared by the recombinant circle PCR[1] technique (*23*). Four plasmids, each coding for a single mutant of glutamate racemase (D10N, D36N, E152Q, and H186N), were obtained and fully sequenced. The mutant enzymes were purified in an identical fashion to the wild-type enzyme (*24*). ESI-MS of the pure enzymes confirmed the predicted molecular mass.

Assay for Glutamate Racemase Activity. The kinetic constants for each mutant enzyme were determined using a circular dichroism (CD) assay (*24*) in both the D-Glu → L-Glu and L-Glu → D-Glu reaction directions. The reactions were performed at 30 °C in 10 mM potassium phosphate buffer, pH 8, with 0.2 mM DTT and glutamate concentrations varying from 1 mM (the lower sensitivity limit of the CD spectrophotometer) up to 5 × K_m. The change in ellipticity at 210 nm was monitored with a JASCO 710 CD spectrophotometer following enzyme addition.

V_{max} Isotope Effects. The V_{max} isotope effects were determined in both reaction directions by comparing the initial velocity of a reaction containing 20 mM of an enantiomer of [2-^{1}H]glutamate to one containing 20 mM of the same enantiomer of [2-^{2}H]glutamate. The ellipticity of a sample (250 μL) equilibrated to 30 °C containing glutamate and 0.2 mM DTT in 10 mM potassium phosphate buffer, pH 8, was monitored at 210 nm following the addition of the appropriate enzyme. The rate was determined from the slope over the first 10% of the reaction.

RESULTS

Sequence Alignment. A primary sequence alignment of the 13 distinct isozymes of glutamate racemase was performed using the CLUSTAL W (1.81) alignment algorithm, and the results are shown in Figure 2. Regions of relatively high homology were found near the N-termini of the enzymes as well as in the sequences surrounding the active site cysteine residues (C73 and C184 in *L. fermenti*). Inspection of the alignment for strictly conserved Asp, Glu, Lys, and His

[1] Abbreviations: PCR, polymerase chain reaction; ESI-MS, electrospray ionization mass spectrometry; CD, circular dichroism; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

FIGURE 2: Multiple sequence alignment of 13 isozymes of glutamate racemase. Strictly conserved residues are indicated by (*); highly conserved residues are indicated by (:); conserved residues are indicated by (.). Catalytic cysteine residues and residues targeted for mutagenesis are boxed. Sources and GenBank accession numbers are as follows: *L. fermentum*, Q03469; *A. aeolicus*, O66662; *A. pyrophilus*, P56868; *H. pylori*, P56068; *L. brevis*, P48797; *P. pentosaceus*, Q08783; *B. sphaericus*, P52972; *S. haemolyticus*, P52974; *B. subtilis*, P94556; *M. leprae*, P46705; *M. tuberculosis*, Q10626; *E. coli*, P22634; *H. influenzae*, P52937.

Table 1: Kinetic Constants for Wild-Type and Mutant Glutamate Racemases D10N, D36N, E152Q, and H186N

	L-glutamate			D-glutamate		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
wild type[a]	69	0.33	2.1×10^5	68	0.26	2.6×10^5
D10N	0.068 ± 0.004[b]	1.3 ± 0.1	$(5.3 \pm 0.3) \times 10^1$	0.063 ± 0.006	1.2 ± 0.2	$(5.3 \pm 0.5) \times 10^1$
D36N	20 ± 3	35 ± 3	$(5.7 \pm 0.8) \times 10^2$	22 ± 1	41 ± 7	$(5.5 \pm 1.4) \times 10^2$
E152Q	38 ± 4	5.8 ± 0.5	$(6.5 \pm 0.1) \times 10^3$	22 ± 1	3.5 ± 0.3	$(6.1 \pm 0.6) \times 10^3$
H186N	0.045 ± 0.004	1.1 ± 0.3	$(4.1 \pm 1.0) \times 10^1$	0.093 ± 0.002	4.5 ± 0.4	$(2.1 \pm 0.1) \times 10^1$

[a] Data taken from (*24*). [b] Errors expressed as standard error of the mean ($n = 3$).

residues led to the identification of four residues that were targeted for mutagenesis (Asp10, Asp36, Glu152, and His186). Asp10 and His186 are located in the areas of highest homology whereas Asp36 and Glu152 are in areas of only modest homology.

Preparation and Characterization of Mutants. The four mutants, D10N, D36N, E152Q, and H186N, were prepared using the recombinant circle polymerase chain reaction (RC-PCR) technique of Jones and Winistorfer (*23*). In each case, an ionizable side chain was replaced with an isosteric non-ionizable side chain. The mutant genes were fully sequenced to ensure that no unintentional mutations were induced during PCR amplification. The mutant proteins were over-expressed in *E. coli* and purified to homogeneity as judged by SDS−PAGE. In each case, electrospray mass spectrometry confirmed that the mass of the enzymes was as expected.

The kinetic constants for the racemization of glutamic acid were followed in both reaction directions using a circular dichroism spectroscopy assay (Table 1) (*24*). The greatest effects on the k_{cat} values were observed with the mutants D10N and H186N, each of which dropped by a factor of 10^3. Only minor decreases in k_{cat} values were observed with the D36N and E152Q mutants. Conversely, the largest perturbations in the K_M values were observed with D36N and E152Q (a 100-fold and an 18-fold increase, respectively), whereas the D10N and the H186N mutants showed only a modest 3-fold increase. In all cases except H186N, the ratio of k_{cat}/K_M values for the forward and reverse reactions was within experimental error of unity, as expected for a racemase obeying the Haldane equation. With H186N, an "iso mechanism" may be operative where interconversion of the two free enzyme forms that differ in protonation state could be

Table 2: V_{max} Isotope Effects for Wild-Type and Mutant Enzymes

	V_{max} isotope effect		
	D-Glu → L-Glu	L-Glu → D-Glu	D→L/L→D RATIO
wild type[a]	3.1 ± 0.5	2.2 ± 0.4	1.4 ± 0.3
D10N	3.7 ± 0.3[b]	1.68 ± 0.02	2.2 ± 0.2[c]
E152Q	4.2 ± 0.3	2.44 ± 0.07	1.7 ± 0.1
H186N	2.9 ± 0.2	4.2 ± 0.1	0.69 ± 0.05

[a] Data taken from (*18*). [b] Errors expressed as standard error of the mean ($n = 3$). [c] Errors are standard errors of the ratio.

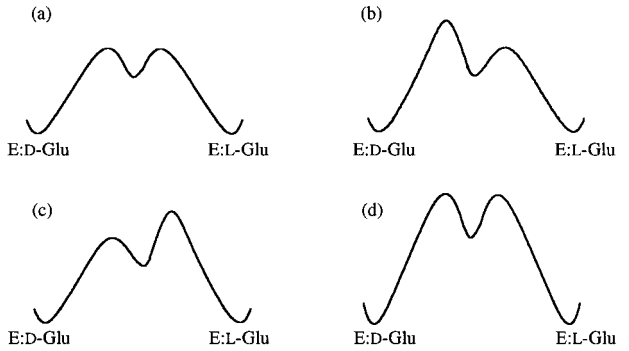

FIGURE 3: Conceptual free energy profiles for the racemization of glutamate by (a) wild-type glutamate racemase, (b) D10N, (c) H186N, and (d) E152Q.

kinetically significant (*25*). This has been observed with proline racemase (*26*) and the serine mutants of glutamate racemase (*21*).

Kinetic Isotope Effect Measurements. To probe whether the mutations induced significant asymmetry into the reaction free energy profile, the primary kinetic isotope effects on the racemization of [2-^{2}H]glutamate were measured in both reaction directions. In previous work, V_{max}/K_M isotope effects were measured using a "competitive deuterium washout" experiment (*18*, *21*). This requires following the complete racemization of an equimolar mixture of the glutamate enantiomers (one of which is deuterated) by circular dichroism spectroscopy. In the cases of mutants with very low activity, however, this is problematic since the high concentrations of protein required to complete the reaction absorb the incident light and cause low signal-to-noise ratios. In addition, significant drifting of the signal occurs over the long time required for data aquisition. Instead V_{max} isotope effects were obtained by comparing the initial velocities of labeled and unlabeled glutamate under identical, saturating conditions (Table 2).

In the wild-type reaction, the V_{max} isotope effect is 3.1 in the D→L direction and 2.2 in the L→D direction. The ratio of the two effects is 1.4 ± 0.3, indicating that neither step is solely rate determining and therefore the reaction free energy profile is somewhat symmetric (Figure 3a). In the case of the D10N mutant, the V_{max} isotope has increased in the D→L direction and has decreased in the L→D direction (3.7 and 1.68, respectively). The resulting ratio of 2.2 ± 0.2 indicates that the barrier to deprotonation of the D-enantiomer has increased relative to that of the L-enantiomer and a greater degree of asymmetry has been introduced by the mutation (Figure 3b). With the H186N mutant, the results were just the opposite in that the V_{max} isotope effect decreased slightly in the D→L direction and increased in the L→D direction (2.9 and 4.2, respectively). The ratio of 0.69 ± 0.05 indicates that the barrier to deprotonation of the D-enantiomer has decreased relative to that of the L-enantiomer (Figure 3c). In the case of the E152Q mutant, both isotope effects increased somewhat, yet the ratio of 1.7 ± 0.1 is similar to that obtained with the wild-type enzyme. This indicates that while the chemical steps were more cleanly rate limiting, no significant asymmetry was induced into the reaction free energy profile (Figure 3d). No isotope effect measurements were made with the D36N mutant since X-ray crystallographic analysis of the racemase structure (*22*) from *Aquifex pyrophilus* strongly suggests it is not located in the active site (see Discussion).

DISCUSSION

The cofactor-independent amino acid racemases and epimerases are mechanistically linked by their common use of two active site cysteine thiols as acid/base catalysts (*1*). Information on the roles of other active site residues, however, remains sparse. In the accepted mechanism for these stereochemical inversions, an initial deprotonation event generates a resonance-stabilized carbanionic intermediate, and reprotonation on the opposite face generates the product (Figure 1). Given that the α-proton of the amino acid is relatively nonacidic (pK_a approximately 21 if bound in a fully protonated form) (*27*, *28*), and that the pK_a of a thiol is around 10, it would seem that the enzyme would need to significantly stabilize the intermediate in order to achieve reasonable reaction rates. One possible interaction that could help to achieve this is a strong hydrogen bond between the carboxylate oxygen of the intermediate and an enzymic acid (*29*, *30*). Another possibility is electrostatic stabilization of the anion via a positively charged residue or metal ion (*31*). An interaction that serves to stabilize the intermediate would also be expected to lower the energy of both transition states to a relatively equal extent. Interruption of such an interaction by mutagenesis could be expected to increase the barrier for both steps of the reaction but not result in significant asymmetry in the free energy profile.

Another possible factor that could serve to increase the rate of deprotonation for a given glutamate enantiomer would be to maximize the amount of corresponding thiolate present in the enzyme−substrate complex. Stabilization of the thiolate could be achieved by hydrogen bonding with the side chain of a neighboring residue (Figure 4A) or via electrostatic interactions with a positively charged residue. An alternative possibility is that a neighboring residue could assist the deprotonation of glutamate by participating in general base catalysis with a neutral cysteine thiol (Figure 4B). The assistance of an adjacent base could help to explain how the Cys-to-Ser mutants retained reasonable levels of enzymatic activity despite the widely differing pK_as between a thiol (p$K_a \approx 10$) and an alcohol (p$K_a \approx 16$), since it would not be necessary to form a discrete alkoxide ion. A similar type of proton relay has been proposed to operate in the reaction catalyzed by UDP−galactose 4-epimerase, where a serine/tyrosinate pair acts as the base during the NAD^+-dependent oxidation of the C-4 hydroxyl (*32*). It is interesting to note that the surprisingly high activity of the Cys-to-Ser mutants of glutamate racemase has also been observed with diaminopimelate epimerase and thus appears to be a general trend among this family of enzymes (*10*). Regardless of the exact nature of the interaction, the mutation of a residue that directly assists a given cysteine to function as a base should introduce asymmetry into the free energy profile of the

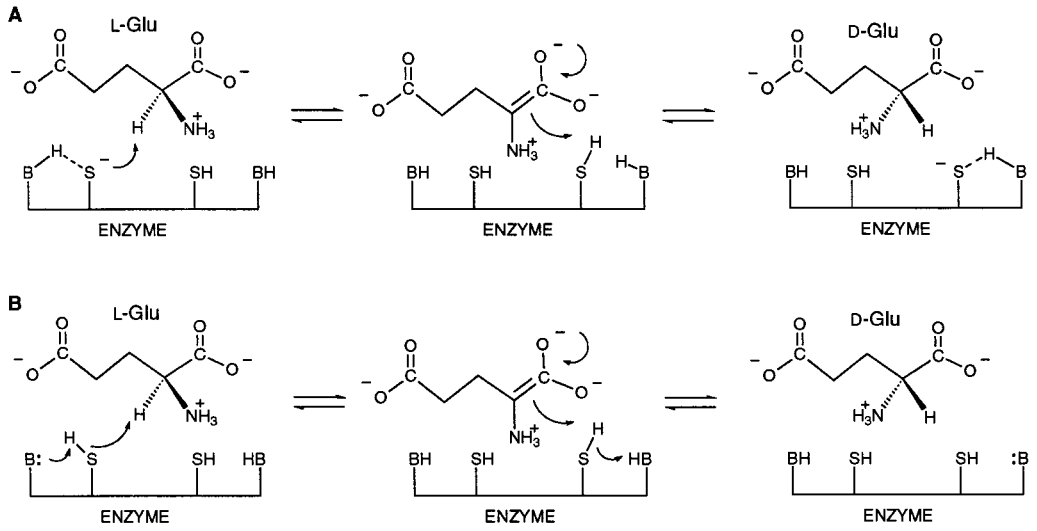

FIGURE 4: Potential roles for residues assisting catalytic acid/base: (A) stabilization of a thiolate by hydrogen bonding or (B) general base assistance involving a neutral thiol.

reaction in a manner analogous to mutations of the cysteine residue itself.

The kinetic constants obtained in this study clearly indicate that Asp10 and His186 are important for catalysis since relatively conservative mutations resulted in dramatic reductions in the value of k_{cat}. Mutations to residues that assist the cysteine bases in the deprotonation of glutamate would be expected to significantly lower the value of k_{cat} since mutations to the cysteine residues themselves (particularly Cys-to-Ala) result in substantially decreased k_{cat} values (*18*−*21*). Glu152 is likely playing a role in substrate binding since the E152Q mutant showed only a modest decrease in the value of k_{cat} whereas the value of K_M for L-Glu increased by 18-fold. The D36N mutant also showed only a modest decrease in k_{cat} and a K_M value for D-Glu that had increased by 100-fold. This is somewhat difficult to interpret since the structure of *Aquifex pyrophilus* glutamate racemase shows that the corresponding D33 is not located in the active site of the enzyme (*22*).

Kinetic isotope effects are useful tools for determining whether asymmetry has been introduced by mutagenesis of racemase active sites (*21*, *33*−*35*). In this study, the isotope effects indicate that asymmetry has been introduced by the mutagenesis of either Asp10 or His186. In the case of the D10N mutant, the ratio of the V_{max} isotope effects (D→L/L→D) has increased with respect to that of wild-type enzyme. This is similar to the results obtained with the C73S enzyme (*21*), indicating that Asp10 may assist Cys73 in functioning as an acid/base residue as outlined in Figure 4. Conversely, with the H186N mutant, the ratio of the V_{max} isotope effects (D→L/L→D) has decreased with respect to that of the wild-type enzyme. This is similar to the results obtained with the C184S enzyme (*21*), indicating that His186 may assist Cys184. A further aspect to consider is that the kinetic constants displayed by the H186N mutant suggested that an "iso mechanism" may be operative and that interconversion of the two free enzyme forms may be kinetically significant. In the isotope effect studies, the basic thiol(ate) is deuterated upon each turnover, and therefore the regeneration of the correct enzyme form could contribute to the measured isotope effect. In the case of proline racemase, this process is completely analogous to the catalytic event with the exception that water replaces bound glutamate (*5*, *6*). If this is also true in glutamate racemase, mutations to the cysteine that acts as the catalytic base (or residues acting in concert with this residue) should affect the transition states for both deprotonation and regeneration of the active enzyme form in a similar manner. Thus, the expected asymmetry in the kinetic isotope effects for these two scenarios should lead to the same mechanistic conclusion. While these interpretations are consistent with the data, it should be mentioned that the extent to which asymmetry was induced in both the D10N and H186N mutants was not as dramatic as observed with the Cys-to-Ser mutants. It is conceivable that any mutation to an active site residue could induce asymmetry into the free energy profile. It should also be noted that the E152Q mutant displayed an increased isotope effect in the D→L direction and essentially no change in the isotope effect in the L→D direction. This could be interpreted as evidence for its involvement in the deprotonation of D-Glu; however, the relatively small degree of induced asymmetry (as indicated by the changes to the ratio of isotope effects) and the modest effect on k_{cat} values make this a less likely candidate for a general base assisting Cys184. It is certainly not possible to rule out the notion that either Asp10 or His186 could be functioning as an acid that protonates, or hydrogen bonds to, the carboxylate of the anionic intermediate. This potential role is analogous to that of Glu317 in the *Pseudomonas putida* mandelate racemase (*29*, *36*, *37*).

The crystal structure of the *A. pyrophilus* glutamate racemase clearly shows that the residues corresponding to C73, C184, D10, H186, and E152 of the *L. fermenti* enzyme are all located in the active site (*22*). The dimeric enzyme was cocrystallized with a weak competitive inhibitor, D-glutamine (K_I = 50 mM vs K_M = 0.5 mM for D-Glu); however, the D-Gln was found to bind in a reverse orientation with the side chain amide located between the two cysteine thiols. For this reason, it is dangerous to extrapolate the observed positions of the side chains to those in a productive substrate/intermediate complex. Nevertheless, the sulfur atom of Cys70 (Cys73 in *L. fermenti*) is located 4.0 Å away from the Oδ1 atom of Asp7 (Asp10 in *L. fermenti*) and is therefore appropriately positioned to allow interactions of the type outlined in Figure 4. The sulfur atom of Cys178 (Cys184 in

L. fermenti) is located 4.2 Å away from the Oϵ1 atom of Glu147 (Glu152 in *L. fermenti*) from the other subunit of the dimeric protein. Our mutagenesis studies have indicated that Glu152 is not essential for catalysis and therefore does not appreciably affect the ability of Cys178 to act as a catalytic acid/base residue. It is also interesting to note that the *L. fermenti* enzyme has been reported to be active as a monomer based on size exclusion chromatography studies (*24*). The distance between the Cys178 sulfur atom and the His180 (His186 in *L. fermenti*) Nδ1 atom is 7.3 Å; however, this distance could easily be reduced to < 4 Å by simple rotations of the side chains. It is interesting to note that a histidine residue and a glutamate residue are also present in the active site of diaminopimelate epimerase (*12*). It has been proposed that these play the role of either electrostatically interacting with charged groups on the substrate or modulating the pK_a values of the active site cysteines. Reasonable candidates for the residue in glutamate racemase that is thought to stabilize the anionic intermediate by hydrogen bonding to the carboxylate group are the strictly conserved Tyr39 and Thr114 (Tyr42 and Thr117 in *L. fermenti*).

Mutagenesis studies were also reported on the *A. pyrophilus* enzyme; however, the results were at odds with ours and somewhat difficult to interpret (*22*). The residues Asp7 and Glu147 (corresponding to Asp10 and Glu152 in *L. fermenti*) were converted to a serine and asparagine, respectively. The values of k_{cat} were reported to drop by factors between 6- and 165-fold depending on the reaction direction, and the values of K_M were relatively unaffected. The authors concluded that both residues were important for catalysis; however, the reported effects were not dramatic. It remains unclear how the rates were measured in the L-Glu to D-Glu direction using a stopped assay involving L-glutamate dehydrogenase (*38*).

In summary, Asp10 and His186 have been shown to play critical roles in the reaction catalyzed by the *L. fermenti* glutamate racemase. Mutating these residues causes marked decreases in the values of k_{cat} and the introduction of asymmetry into the reaction free energy profile. This is consistent with their proposed roles in assisting the active site cysteines to function as acid/base catalysts.

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