Catalytic Acid/Base Residues of Glutamate Racemase†

Suzana Glavas and Martin E. Tanner*

Department of Chemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z1, Canada

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ABSTRACT: Glutamate racemase is a cofactor-independent enzyme that employs two active-site cysteine residues as acid/base catalysts during the interconversion of glutamate enantiomers. In a given reaction direction, a thiolate from one of the cysteines abstracts the α-proton, and the other cysteine thiol delivers a proton to the opposite face of the resulting carbanionic intermediate. This paper reports that the C73S and C184S mutants are still capable of racemizing glutamate with specificity constants about 10^3-fold lower than those of the wild-type enzyme. A "one-base requiring" reaction, the elimination of water from *N*-hydroxyglutamate, has been used to deduce which thiol acts as the base for a given enantiomer. With D-*N*-hydroxyglutamate the C73S mutant is a much poorer catalyst than wild-type enzyme, whereas the C184S mutant is a somewhat better catalyst. This trend was reversed with L-*N*-hydroxyglutamate, suggesting that Cys73 is responsible for the deprotonation of D-glutamate and Cys184 is responsible for the deprotonation of L-glutamate. In addition, with C73S the V_{max}/K_M isotope effect on D-glutamate racemization was greater than that seen with wild-type enzyme, whereas the isotope effect with L-glutamate had decreased. The results were reversed with the C184S mutant. This is interpreted as being due to an asymmetry in the free energy profiles that is induced upon mutation, with the deprotonation step involving a serine becoming the more cleanly rate-determining of the two. These results support the above assignment and the notion that a carbanionic intermediate is formed during catalysis.

The enzyme glutamate racemase (MurI, EC 5.1.1.3) catalyzes the interconversion of the enantiomers of glutamic acid and provides bacteria with a source of D-glutamate. D-Glutamate is used in the construction of peptidoglycan, which is the rigidifying component of the bacterial cell wall and protects the organism from osmotic lysis (*1*). Enzymes responsible for peptidoglycan biosynthesis are attractive targets for drug design since many clinically used antibiotics act on this pathway (*2*).

All known amino acid racemases and epimerases ultimately employ a mechanism that involves the deprotonation of the amino acid at C-2, followed by the reprotonation of the resulting carbanionic intermediate in the opposite stereochemical sense (*3*, *4*). These enzymes face the difficult task of removing the α-protons of amino acids that are estimated to have pK_as as high as 21 (for the fully protonated form of the amino acid) (*5*). The well-studied enzyme alanine racemase overcomes this hurdle through the use of a pyridoxal phosphate cofactor (PLP)[1] (*6*, *7*). The amine of the substrate is first attached to the cofactor via an imine linkage. The cofactor serves as an electron sink that acidifies the α-proton and allows the inversion to proceed via a deprotonation/reprotonation mechanism.

The mechanism employed by glutamate racemase is of interest because it is among a group of enzymes, including proline racemase (*8*−*11*), diaminopimelate epimerase (*12*−*14*), and aspartate racemase (*15*), that operate in a cofactor-independent fashion. Mechanistic studies on glutamate racemase show that the reaction in ^2H_2O proceeds with the incorporation of solvent-derived deuterium at C-2 and that the racemization of either enantiomer of [2-^{2}H]glutamate is slowed by a primary kinetic isotope effect (*16*, *17*). These observations support a deprotonation/reprotonation mechanism somewhat analogous to that of alanine racemase however, it is difficult to explain how the carbanionic intermediate is stabilized without the aid of covalent catalysis via a PLP cofactor.

Further studies showed that glutamate racemase uses two active-site cysteine residues in promoting this 1,1-proton transfer process (Figure 1). It appears that the thiolate of one cysteine serves to deprotonate the substrate, and the thiol of the second cysteine protonates the carbanionic intermediate in the opposite stereochemical sense. Experiments with deuterated substrates showed that the initially formed products contain exclusively solvent-derived isotope at C-2, whereas recovered starting materials contain exclusively substrate-derived isotope (in either reaction direction) (*18*, *19*). This is consistent with a "two-base" mechanism involving monoprotic bases that do not undergo proton exchange with solvent during the lifetime of the carbanionic intermediate.

More direct evidence for cysteine participation was obtained when these residues were independently mutated to alanine or threonine and the resulting proteins were found to be inactive (*17*, *20*). In the case of the *Lactobacillus fermenti* enzyme, one of the inactive alanine mutants was

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* Corresponding author: phone: 604-822-9453; FAX 604-822-2847; e-mail mtanner@chem.ubc.ca.

[1] Abbreviations: PLP, pyridoxal phosphate; NADH, dihydronicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane; Trien, triethanolamine; DTT, dithiothreitol; PCR, polymerase chain reaction; CD, circular dichroism; NMR, nuclear magnetic resonance; ESI-MS, electrospray ionization mass spectrometry; GC, gas chromatography; DCI, desorption chemical ionization.

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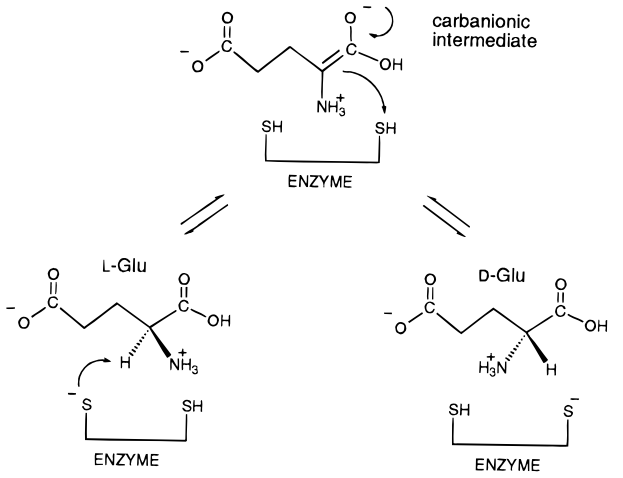

FIGURE 1: Proposed mechanism for the reaction catalyzed by glutamate racemase.

still capable of promoting the elimination of HCl from a single enantiomer of *threo*-3-chloroglutamate, whereas the other mutant could promote the elimination only with the opposite enantiomer (*17*). These studies showed that the active site of the mutants was still capable of catalyzing a reaction requiring only a single enzymic base and that a given cysteine assists in the deprotonation of a given enantiomer. Additional evidence was found with the use of the affinity label "aziridino-glutamate", which alkylates an active-site cysteine residue and establishes that the thiolate is in the proper vicinity to directly deprotonate bound glutamic acid (*21*).

In this paper we describe the preparation of, and studies on, the cysteine-to-serine single mutants of recombinant *Lactobacillus fermenti* glutamate racemase. We have found that these mutants are still capable of racemizing glutamate with specificity constants about 10^3-fold lower than those of the wild-type enzyme. We have also used the alternate substrate *N*-hydroxyglutamate as well as primary kinetic isotope effect measurements to show that the mutations endowed these enzymes with an asymmetrical free energy profile. This provides indirect evidence for the existence of a carbanionic intermediate and allows us to predict which of the cysteine residues is responsible for the deprotonation of a given glutamate enantiomer.

EXPERIMENTAL PROCEDURES

Materials. All reagents were purchased from Sigma or Aldrich. Ultrapure potassium phosphate (99.99%) and ultrapure potassium hydroxide (99.99%) were purchased from Aldrich. Wild-type glutamate racemase was purified from *Escherichia coli* DH5α bearing the pKG3 vector as described previously (*22*). Protein concentrations were determined by the method of Bradford (*23*) with bovine serum albumin as the standard.

D- and L-[2-^{2}H]Glutamate were prepared and analyzed according to the method of Tanner et al. (*17*) as follows. (D,L)-[2-^{2}H]-*N*-Acetylglutamate was prepared by the enzymatic racemization of glutamate in D_2O followed by *N*-acetylation (Note: an error was found in the experimental section of ref *17*; 6 M NaOH is used in the acylation, not 6 M HCl). Treatment with porcine kidney acylase I yielded L-[2-^{2}H]glutamate, and acid hydrolysis of the remaining D-*N*-acetyl-[2-^{2}H]glutamate gave D-[2-^{2}H]glutamate. The enantiomeric purity of each sample was evaluated by conversion to the dimethyl ester and derivatization with (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride followed by GC analysis on an HP-17 GC column. The deuterium content of the derivative was established by DCI mass spectroscopy.

D- and L-*N*-Hydroxyglutamate were synthesized by the method of Polonski and Chimiak (*24*) with the modifications described by Glavas and Tanner (*25*). The enantiomeric purity of the *N*-hydroxyglutamate samples was determined by reduction to the corresponding glutamate enantiomers (*24*) followed by an enzymatic analysis for both total glutamate and L-glutamate (*25*).

Synthesis of Oligonucleotides. Oligonucleotides were synthesized on a PE Applied Biosystems Model 380B DNA synthesizer followed by deprotection and ammonia−butanol purification (Nucleic Acid Protein Service Unit, UBC). Concentrations were determined by the absorbance at 260 nm. The following oligonucleotides were synthesized: (1) GGTCGTGGCCTCCAATACGGCGA, (2) GATTATGGGCTCCACCCACTTCC, (3) TCGCCGTATTGGAGGCCACGACC, (4) GGAAGTGGGTGGAGCCCATAATC, (5) CGACGAGCGTGACACCACGATGCC, and (6) GCAGAGCGAGGTATGTAGGCGGTGC. Primers 1, 2, 3, and 4 are complementary to the glutamate racemase gene. Underlined regions code for the Cys−Ser mutation. Primers 5 and 6 are complementary to the pUC18 vector.

Site-Directed Mutagenesis and Mutant Purification. Plasmids coding for the mutant enzymes were prepared by the recombinant circle PCR technique described by Jones and Winistorfer (*26*). The pKG3 vector bearing the glutamate racemase gene was linearized in two separate reactions with either *Eco*RI (Promega) or *Hin*dIII (Gibco-BRL). Two PCR amplifications were performed on the *Eco*RI-linearized DNA: reaction 1 (C73S) contained primers 1 and 6, and reaction 2 (C184S) contained primers 2 and 6. The *Hin*dIII-linearized DNA was amplified with the following primer pairs: reaction 3 (C73S) used primers 3 and 5, and reaction 4 (C184S) used primers 4 and 5. The combined products of reactions 1 and 3 (C73S) and reactions 2 and 4 (C184S) were denatured (94 °C for 3 min) and annealed (50 °C for 2 h) prior to transformation into $CaCl_2$-competent *E. coli* DH5α. Individual colonies that grew on LB−agar plates supplemented with 50 μg/mL ampicillin were used to inoculate a 5 mL overnight culture of LB containing 50 μg/mL ampicillin. Plasmid DNA was isolated with a Wizard Miniprep DNA purification system (Promega). Two plasmids containing the correct mutation [pSG01 (C73S) and pSG02 (C184S)] were obtained and the entire gene was sequenced to ensure no other errors were present.

The mutant proteins were overexpressed and purified in an identical fashion to the wild-type enzyme (*22*). In each case, ESI-MS was used to confirm that the proteins had the expected molecular mass.

Assay for Glutamate Racemase Activity. Routine kinetics and phosphate inhibition studies were performed at pH 8 in the D-Glu to L-Glu direction using a known coupled enzyme assay (*22*) that employs L-glutamate dehydrogenase/NAD^+ and diaphorase/*p*-iodonitrotetrazolium violet (50 mM Trien-HCl, pH 8). A previously described (*22*) circular dichroism assay (10 mM potassium phosphate, pH 8) was used to obtain

Table 1: Kinetic Constants for Wild-Type and Mutant Glutamate Racemase at pH 8

	wild type[a]		C73S		C184S	
	L-Glu	D-Glu	L-Glu	D-Glu	L-Glu	D-Glu[c]
k_{cat} (s^{-1})	69	68	0.19 ± 0.01[b]	1.23 ± 0.04	0.75 ± 0.01	1.95 ± 0.10
K_m (mM)	0.33	0.26	1.2 ± 0.2	5.3 ± 0.7	2.1 ± 0.3	4.8 ± 1.3
k_{cat}/K_m (s^{-1} M^{-1})	2.1×10^5	2.6×10^5	$(1.6 \pm 0.4) \times 10^2$	$(2.3 \pm 0.4) \times 10^2$	$(3.6 \pm 0.6) \times 10^2$	$(4.1 \pm 1.2) \times 10^2$

[a] Data taken from ref *22*. [b] Errors expressed as standard error of the mean ($n = 3$). [c] $n = 4$.

the kinetic parameters when measured in both reaction directions. The value of K_M obtained in this fashion was corrected for competitive inhibition by phosphate using K_I values (5.5 mM and 24 mM for C73S and C184S, respectively) measured as described above.

pH Dependence of k_{cat}/K_m. The pH dependence of the k_{cat}/K_m values for the racemization of D-glutamate by wild-type or mutant enzymes was determined by following the reaction to completion at a single low substrate concentration (0.03 mM). The coupled enzyme assay described above was used with the exception that 50 mM Tris-HCl (pH 7−9) replaced the Trien-HCl as the buffer. To ensure the reaction was coupled at all pHs, 4 units of diaphorase and 67.5 units of L-glutamate dehydrogenase was added. Under these conditions the D-glutamate is consumed with a rate constant of $k_{obs} = [E]_0 k_{cat}/K_M$. Data were fitted to a first-order rate equation and k_{obs} was obtained from the graphics program GraFit.

Measurement of Second-Order Rate Constant for N-Hydroxyglutamate Reactions. The specificity constants for the conversion of D- and L-*N*-hydroxyglutamate into α-ketoglutarate were measured by a coupled enzyme assay that employs L-glutamate dehydrogenase, NH_4Cl, and NADH as described previously (*25*). The progress of the reaction at a single low *N*-hydroxyglutamate concentration (0.02 mM) was followed to completion and under these conditions the *N*-hydroxyglutamate is consumed with a rate constant of $k_{obs} = [E]_0 k_{cat}/K_M$. The time-dependent decrease in NADH concentration measured at 340 nm was analyzed as a first-order exponential decay by the graphics program, GraFit.

Competitive Deuterium Washout Experiment. The competitive deuterium washout experiment was performed as previously described (*17*). The CD signal of a solution (250 μL of 10 mM potassium phosphate buffer, pH 8, and 0.2 mM DTT) containing 5 mM deuterated substrate and 5mM undeuterated enantiomer was monitored at 210 nm and 30 °C after addition of 0.07 mg of the appropriate mutant enzyme. The molar ellipticity of glutamate at 210 nm was taken as 23.1 mdeg/(mM·cm).

Double Competitive Deuterium Washout Experiment. The double competitive deuterium washout experiment was performed as previously described (*17*). The CD signal of a solution (250 μL of 10 mM potassium phosphate buffer, pH 8, and 0.2 mM DTT) containing equal amounts of each deuterated enantiomer (6 mM each for C184S and 2.5 mM each for C73S) was monitored at 210 nm and 30 °C following the addition of 0.07 mg of enzyme.

RESULTS

Preparation and Kinetic Evaluation of the C73S and C184S Glutamate Racemase Mutants. The recombinant *L. fermenti* glutamate racemase used in this work contains only the two catalytic cysteine residues, C73 and C184, and is distinct from the *E. coli* enzyme as it is not regulated by UDP-MurNAc-L-Ala (*20*, *27*). PCR-based site-directed mutagenesis was used to prepare overexpression plasmids containing the genes for the C73S and C184S mutants. Complete sequencing of the inserts confirmed that only the desired mutations had been introduced. Expression in *E. coli* and purification by ion-exchange chromotography produced the mutant proteins of the expected molecular weight as determined by ESI-MS. Glutamate racemase activity can be accurately measured in the D-Glu to L-Glu direction by using a spectrophotometric coupled enzyme assay involving L-glutamate dehydrogenase and diaphorase (*22*). To measure the activity in both reaction directions, a circular dichroism assay must be employed however, the errors in the kinetic constants can be as high as 20% due to the lack of sensitivity of this assay method (*22*). The kinetic constants for both the wild-type and mutant enzymes were determined in both reaction directions and under identical conditions by the CD assay (Table 1). In the case of the mutant enzymes, the values of K_M were corrected for the presence of 10 mM phosphate buffer, which acts as a competitive inhibitor. It is surprising to note that phosphate does not significantly inhibit the wild-type enzyme (*22*), suggesting there is a direct interaction between the bound phosphate and the hydroxyl groups of the serine mutants. In both cases the mutants retained significant levels of racemase activity, indicating that the serine residues were capable of acting as acid/base catalysts in this reaction. This was particularly noticeable with the C184S mutant, where the value of k_{cat} for the racemization of D-Glu was 1.95 s^{-1} as compared to 68 s^{-1} for the wild-type enzyme. The values of K_M increased roughly 10-fold in all cases, indicating that the mutations had affected the ability to bind glutamate and that the activity was not due to a contamination of the wild-type enzyme. The values of k_{cat}/K_M were essentially equivalent in both reaction directions (at pH 8), as predicted by the Haldane equation for a racemase reaction.

The pH dependence of the k_{cat}/K_M value for the racemase was investigated from pH 7−9 by using the coupled enzyme assay (Figure 2A). The pH sensitivity of the coupling enzyme, L-glutamate dehydrogenase, prevented measurements outside this window and the CD assay was too insensitive for use at very low concentrations of glutamate. The racemization of dilute samples of D-Glu (0.03 mM) were followed to completion at various pH values. Under these conditions the observed rate obeys a first-order dependence on D-Glu concentration with $k_{obs} = [E]_0 k_{cat}/K_M$, and the titrations reflect pK_a values of the free enzyme (*28*). The resulting curve is bell-shaped, as expected for an enzyme that must employ both acid and base catalysis, and the maximum rate was observed at pH 8.0. Similar measure-

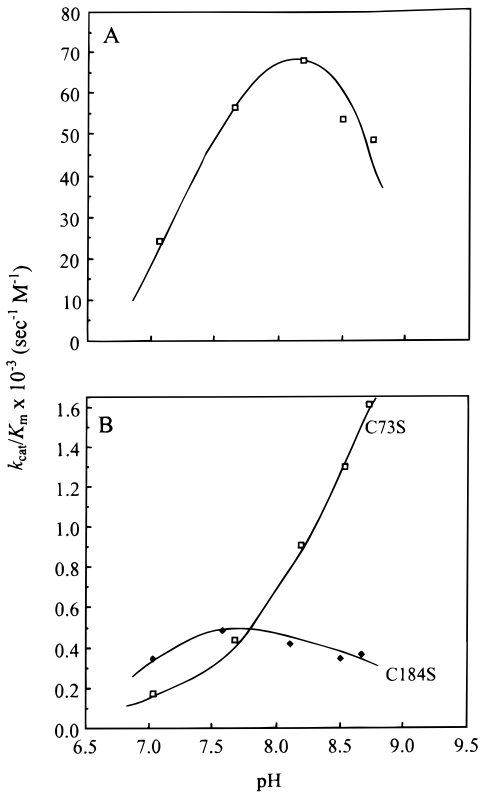

FIGURE 2: Partial pH−rate profile for the racemization of D-glutamate by (A) wild-type glutamate racemase and (B) the C73S and C184S mutants.

ments were made with the C73S mutant and the C184S mutant (Figure 2B) and notable differences were observed. With C73S, the pH−rate profile showed a continual increase from pH 7 to 9, indicating that the pH of maximum rate had shifted to a value of 9 or greater. With C184S, the pH−rate profile showed little variation within this region.

Reactivity of Mutants with N-Hydroxyglutamate Enantiomers. Previous studies showed that D-*N*-hydroxyglutamate acts as a competitive inhibitor of glutamate racemase with a K_I value of 56 μM (*25*). In addition, it is gradually converted to α-ketoglutarate and ammonia (K_M = 57 μM, k_{cat}/K_M = 3.2 × 10^3 M^{-1} s^{-1}). It appears that the enzyme catalyzes the elimination of water from this compound to form an imine, which mimics the carbanionic intermediate and binds well in the active site (Figure 3, X = Y = S). Release of the imine results in hydrolysis and formation of α-ketoglutarate. It was also found that L-*N*-hydroxyglutamate was a substrate for the enzyme-catalyzed elimination, but it was converted to α-ketoglutarate at a much slower rate (k_{cat}/K_M = 30 M^{-1} s^{-1}).

It is reasonable to suspect that only one of the cysteine thiols is involved in promoting the elimination of water from a given enantiomer of *N*-hydroxyglutamate. If this is indeed the case, then the serine mutants we have prepared should appear crippled only with respect to one of the two enantiomers (Figure 3, X = O, Y = S). The specificity constants for both of the elimination reactions were therefore measured with each mutant by the coupled assay described previously (*25*). With D-*N*-hydroxyglutamate as a substrate we found that the C73S mutant is a much poorer catalyst than the wild-type enzyme, whereas the C184S mutant is a somewhat better catalyst (Table 2). This suggests that C73

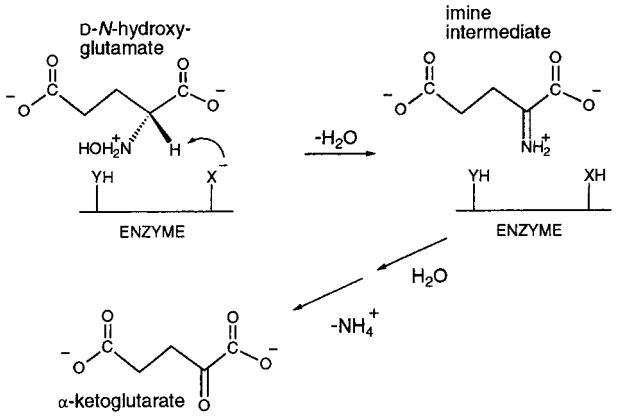

FIGURE 3: Proposed mechanism for the inhibition of glutamate racemase by D-*N*- hydroxyglutamate (wild type, X = Y = S; C73S, X = O, Y = S; C184S, X = S, Y = O).

Table 2: Kinetic Constants for *N*-Hydroxyglutamate Reactions

	k_{cat}/K_m (s^{-1} M^{-1})	
	D-*N*-hydroxyglutamate	L-*N*-hydroxyglutamate
wild type	3.2 × 10^3	30
C73S	58	160
C184S	4.6 × 10^3	<4

is responsible for promoting the elimination reaction with the D-enantiomer and that C184 is not involved. The small increase in the specificity constant for C184S may be attributed to a slightly enlarged cavity that can more easily accommodate the extra bulk of the hydroxylamine moiety. When L-*N*-hydroxyglutamate was tested as a substrate, the results were reversed in that C73S mutant was now a better catalyst than the wild-type enzyme, whereas the C184S mutant was a poorer catalyst. These results support the idea that each cysteine is responsible for the deprotonation of a single enantiomer and lead one to suspect that Cys73 serves to deprotonate D-Glu (with Cys184 deprotonating L-Glu) in the wild-type racemization reaction.

Primary Kinetic Isotope Effects with the Mutant Enzymes. The studies with *N*-hydroxyglutamate provided evidence as to what the role of each cysteine was in the racemization reaction. These studies, however, require the use of an alternate substrate and an elimination reaction and may not accurately reflect what is taking place in the normal racemization reaction. A much more subtle modification is to introduce an isotopic substitution into glutamate itself and monitor the resulting effects on the rate of racemization.

With the wild-type *L. fermenti* racemase, primary kinetic isotope effects are observed in both reaction directions when [2-^{2}H]glutamate is used as the substrate (*17*). This indicates that the reaction profile is somewhat symmetrical and that both deprotonation and reprotonation are partially rate-determining in a given reaction direction. The introduction of a mutation that would affect only one of the two barriers would likely skew this profile such that one of the two steps would now be more cleanly rate-determining. This could easily be observed by changes in the magnitudes of the isotope effects for the mutants.The deuterated enantiomers of glutamic acid were prepared by the racemization of glutamic acid in ^2H_2O, followed by a resolution with the use of porcine kidney acylase. Both were obtained in >98% enantiomeric excess and with a deuterium content of >97%

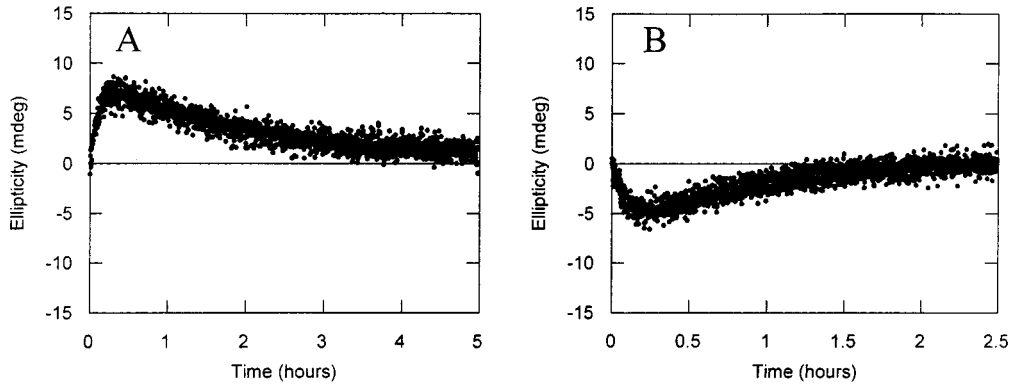

FIGURE 4: Ellipticity at 210 nm in the competitive deuterium washout experiments with C184S: (A) L-[2-^{2}H]glutamate and D-glutamate; (B) D-[2-^{2}H]-glutamate and L-glutamate.

Table 3: V_{max}/K_m Isotope Effects

reaction direction	wild type[a]	C73S	C184S
D-Glu → L-Glu	3.4	5.1 ± 0.3[b]	2.3 ± 0.1
L-Glu → D-Glu	2.5	1.85 ± 0.01	4.80 ± 0.02
ratio	1.35	2.76	0.48

[a] Data from ref 17. [b] Errors are standard errors of the mean ($n = 3$).

at the C-2 position. The isotope effects were determined with the use of a "competitive deuterium washout experiment" that was employed by Albery and Knowles (*8*, *29*) on proline racemase and is analogous to the equilibrium perturbation method described by Cleland (*30*−*32*). In this experiment equimolar amounts of a deuterated substrate and its non-deuterated enantiomer are combined, and the enzymatic racemization in H_2O is followed by CD spectroscopy (210 nm) as a function of time (Figure 4 shows results obtained with C184S). Initially, the observed ellipticity in the mixture is zero since the deuterium substitution does not affect the molar ellipticity of the substrate. As the reaction proceeds, the inversion of the deuterated substrate is slowed by an isotope effect and therefore it accumulates. As a result, the observed ellipticity is perturbed toward that of the deuterated enantiomer (in a positive sense when the L-enantiomer is deuterated; see Figure 4A). Eventually, all of the isotopic label is "washed out" into solution and the resulting racemic mixture of glutamate once again displays a net ellipticity of zero. For a two-base racemase that incorporates solvent isotope only into the product enantiomer, the magnitude of the maximum perturbation in ellipticity is solely a function of the isotope effect on the deuterated enantiomer. A mathematical treatment of the washout experiment can be found in the work of Albery and Knowles on the related enzyme proline racemase (*29*). By use of "composite" rate constants, eq 1 relates the size of the maximum perturbation, λ_{max} (expressed as a mole fraction of the total glutamate), to the V_{max}/K_M isotope effect, $^Hk_{1,2,3}/^Dk_{1,2,3}$:

$$2\lambda_{max} = (R - 2)R^{R/(1-R)} \quad (1)$$

where $R = 2^Hk_{1,2,3}/^Dk_{1,2,3}$. The washout experiments were performed with each mutant in each reaction direction and the resulting V_{max}/K_M isotope effects were compared to those obtained with the wild-type enzyme (Table 3). It is clear that the mutation of Cys73 to serine resulted in an increased isotope effect for the racemization of D-glutamate (5.1 vs 3.4) and a decreased isotope effect for the racemization of L-glutamate (1.85 vs 2.5) when compared to the data obtained with wild-type enzyme. Mutation of the Cys184 residue however, gave completely the opposite results (2.3 vs 3.4 for D-glutamate and 4.8 vs 2.5 for L-glutamate).

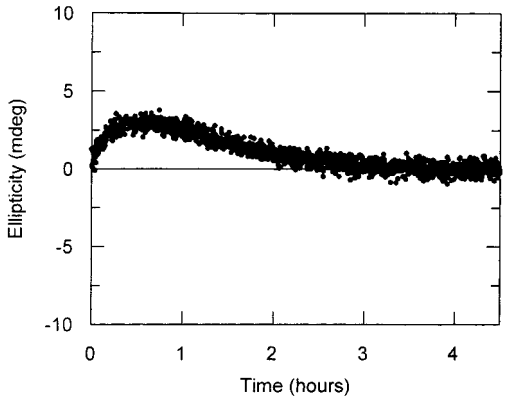

FIGURE 5: Ellipticity at 210 nm in the double competitive deuterium washout experiment with C184S and D,L-[2-^{2}H]-glutamate.

These results were cross-checked via the use of "double competitive deuterium washout experiments". In this experiment racemic deuterated glutamic acid is treated with the racemase in H_2O. Now both enantiomers are initially deuterated and the one that accumulates will be the one whose racemization is slowed by a larger isotope effect. Again a transient perturbation is observed in the ellipticity of the mixture and eventually the value returns to zero when all of the deuterium has been "washed out" into solution (Figure 5 shows results obtained with C184S). The maximum size of the perturbation is dictated by the ratio of the corresponding isotope effects and this ratio can be calculated (*29*) using eq 2:

$$2\lambda_{max} = (1 - \xi)\,\xi^{\xi/(1-\xi)} \quad (2)$$

where ξ is the ratio of the V_{max}/K_M isotope effects. When this experiment was performed with C73S and C184S, the observed ratios of the isotope effects for D-glutamate to that for L-glutamate were found to be 2.8 and 0.52, respectively. These values agree well with those calculated from the individual isotope effects (Table 3).

DISCUSSION

The cofactor-independent amino acid racemases serve as unusual examples of enzymes that employ cysteine residues

in the deprotonation of fairly nonacidic protons ($pK_a \approx 21$). In a given reaction direction, one cysteine thiolate deprotonates the amino acid and a second cysteine thiol donates a proton to the opposite face of the resulting carbanionic intermediate (Figure 1). In the opposite reaction direction the roles of the two cysteines are reversed. In this work the active-site cysteines of glutamate racemase from *L. fermenti* were individually changed to serine residues and the resulting mutant enzymes were found to retain substantial racemase activity. From a consideration of the differences in pK_as of a free thiol ($pK_a \approx 10$) as compared to a hydroxyl ($pK_a \approx 16$), one might expect that the value of k_{cat}/K_M for the mutant-catalyzed racemizations would be decreased dramatically. In fact it was found that the values of k_{cat}/K_M for the C73S and C184S mutants were only decreased by factors of 1.2×10^3 and 6×10^2, respectively. If the free enzyme/mutant must possess a thiolate/alkoxide for binding and catalysis to take place, the amount of mutant enzyme present in the correct protonation state would be decreased by roughly 6 orders of magnitude. This could be somewhat compensated for, however, by the increased efficiency of an alkoxide to act as a base and an overall 10^3-fold rate decrease is not unreasonable (assuming a Brønsted β value of 0.5 for the deprotonation of glutamate). Alternatively, the involvement of a catalytic diad may explain the mutant reactivity. A second residue (such as an imidazole or carboxylate group) could serve as a general base and assist the serine alcohol in the deprotonation step. Such residues may play a similar role in the wild-type reaction with a thiol acting as base or simply serve to stabilize the thiolate anion via H-bonding (in their protonated forms).

The pH$-k_{cat}/K_M$ profile of the wild-type enzyme showed a relatively narrow bell-shaped curve centered at pH 8 (Figure 2A). This is consistent with an enzyme that employs two general acid/base residues of similar pK_a values during catalysis, one of which must be in a protonated form and one of which must be in a deprotonated form for a given reaction direction. This has also been observed in the case of proline racemase (*11*) and is consistent with the observation that the two enzyme forms (differing in the respective protonation states of the bases) will bind only one of the two enantiomers (*10*, *33*). The pK_as likely represent those of the cysteine thiols that participate in the deprotonation−reprotonation steps (Figure 1). The pH−rate profile for the C73S mutant in the D-Glu-to-L-Glu direction shows a steady increase in rate across the accessible pH window with a maximum value at a pH > 9. In this reaction direction, the serine hydroxyl must act as a base but exists primarily in a protonated form. The sharp increase in rate with pH is likely due to the increasing amounts of alkoxide present at higher pH. The C184S mutant showed little pH dependence; presumably because the serine hydroxyl is largely protonated over this region and this residue is required to act as a general acid. We expect that the profiles will be reversed if measured in the L-Glu-to-D-Glu direction. This would require, however, that the ratio of the k_{cat}/K_M values in the two reaction directions would not be unity at most pH values (as is expected for a racemase reaction). This can be understood by considering an "iso mechanism" in which there are two different enzyme forms whose interconversion is partially rate-determining (*34*). In this situation the Haldane equation contains terms reflecting the enzyme interconversion and the ratio of the k_{cat}/K_M values could be nonunity. For the mutant racemase reactions the alkoxide/thiol form of the enzyme would be much less stable than the alcohol/thiolate form and the interconversion between them could be partially rate-limiting. Evidence for a partially rate-limiting interconversion of enzyme forms has been observed in the case of wild-type proline racemase (*35*).

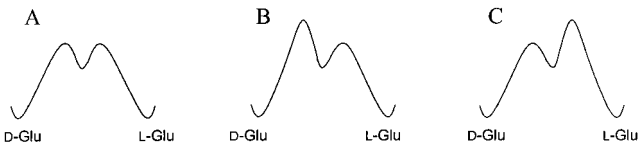

FIGURE 6: Conceptual free energy profiles for the racemization of glutamate: (A) wild-type enzyme; (B) C73S; (C) C184S.

The roles of the active-site cysteine thiols may be examined independently of one another by employing a probe reaction that only requires one enzymic acid/base catalyst. The elimination of water from *N*-hydroxyglutamate (*25*) appears to be such a reaction (Figure 3). The C184S mutant handled the D-enantiomer as well as, if not better than, the wild-type enzyme yet showed a dramatic decrease in its ability to process the L-enantiomer. Conversely, the C73S mutant accepted the L-enantiomer at wild-type rates but was almost inactive toward the D-enantiomer. Since only one of the elimination reactions is affected by a given mutation, the bases must be operating independently and presumably general acid catalysis is not required in the loss of hydroxide. If one assumes that the enantiomers of *N*-hydroxyglutamate bind in a fashion analogous to that of the corresponding glutamate enantiomers, one can conclude that Cys73 is responsible for the deprotonation of D-glutamate (or D-*N*-hydroxygluamate) and that Cys184 is responsible for the deprotonation of L-glutamate (or L-*N*-hydroxyglutamate). These results are in agreement with those found previously with alanine mutants of glutamate racemase (*17*). The C184A mutant was found to catalyze the elimination of HCl from only one enantiomer of *threo*-3-chloroglutamate, and the C73A mutant handled only the opposite enantiomer. Since glutamate−oxalacetic transaminase (an L-glutamate handling enzyme) catalyzed the same reaction as the C73A, it was concluded that Cys 73 is responsible for the deprotonation of the D-enantiomer.

The use of alternative substrates in assigning the roles of the active-site bases is complicated by the fact that these compounds may bind in the active site somewhat differently than the enantiomers of glutamic acid. A more subtle modification that can lead to a less ambiguous conclusion is to use deuterated substrates and observe the changes in the primary kinetic isotope effects that the mutations cause. It is known that the racemization of either enantiomer of [2-^{2}H]glutamic acid is slowed by a modest primary kinetic isotope effect (V_{max}/K_M isotope effects are 2.5 for L-Glu and 3.4 for D-Glu) (*17*). Since isotope effects are seen in each reaction direction, both protonation and deprotonation must be partially rate-determining for the racemization of a given enantiomer and we can infer that the reaction energy profile is somewhat symmetric (Figure 6A). By mutating a single cysteine into a poorer acid/base catalyst, we should perturb this situation and introduce a significant asymmetry into the reaction energy profile (Figure 6B,C). For example, the C73S

mutation is expected to increase the barrier toward the deprotonation of D-glutamate but not that for deprotonation of L-glutamate (Figure 6B). This would mean that deprotonation of D-glutamate would be more cleanly rate-determining and a larger intrinsic isotope effect would be observed. Conversely, the isotope effect on L-glutamate would be masked by a rate-determining step (protonation of the intermediate) that does not involve transfer of the isotopically labeled proton, and therefore the isotope effect should decrease.

The kinetic isotope effects for both mutants were determined in both reaction directions and compared to those seen with the wild-type enzyme. With the C73S mutant the isotope effect on the racemization of D-glutamate (5.1) was greater than that seen with wild-type enzyme (3.4), whereas the isotope effect on the racemization of L-glutamate had decreased (1.85 vs 2.5). This is consistent with the profile outlined in Figure 6B and indicates that Cys73 is responsible for the deprotonation of D-glutamate. The opposite trend was observed with the C184S mutant, indicating its reaction profile resembles that in Figure 6C and demonstrating that Cys184 is responsible for the deprotonation of L-glutamate. It is possible that the increases in the isotope effects may simply be the result of changing a sulfur (with a low fractionation factor) for an oxygen (normal fractionation factor) and thereby changing the intrinsic isotope effect on that elementary step. One would not, however expect a corresponding decrease in the isotope effect in the reverse reaction direction if that were the sole factor at play. The asymmetric changes in the isotope effects also support the notion that a carbanionic intermediate exists and that the deprotonation/reprotonation process occurs in a stepwise fashion with two distinct reaction barriers (as implied in Figure 6).

Similar experiments have been performed on mandelate racemase, a two-base enzyme that utilizes a lysine and a histidine residue to catalyze an analogous stereochemical inversion (*3*, *4*). The lysine acts as the base in the deprotonation of (*S*)-mandelate, and mutation of this residue to arginine was demonstrated to create an asymmetry in the free energy profile (*36*). The isotope effect of 3.6 on the racemization of (*S*)-mandelate did not change significantly with the mutation; however, the isotope effect with (*R*)-mandelate fell from 3.2 to unity (*30*). Mutation of the histidine to asparagine gave a protein with no detectable racemase activity; however, a "one-base-requiring" reaction, the elimination of bromide from *p*-(bromomethyl)mandelate, was shown to proceed only with the (*S*)-enantiomer and the (*R*)-enantiomer was no longer handled (*37*). These observations were used to assign the roles of the bases and agree well with the crystallographic data available on this enzyme.

Further research from this laboratory will address the possibility that a second set of acid/base catalysts are involved in aiding the thiol(ate)s during catalysis. Four residues (D10, D36, E152, and H186 in *L. fermenti*) are conserved among all known glutamate racemases and will serve as an initial target for mutagenesis experiments. Similar asymmetric free energy profiles should result upon mutation of a residue functioning in this fashion, and kinetic isotope effect measurements will be used to probe these effects.

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