Isotope Effects and the Identification of Catalytic Residues in the Reaction Catalyzed by Glutamate Racemase†

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ABSTRACT: Primary kinetic isotope effects on V_{max} were observed in both reaction directions upon racemizing samples of [2-^{2}H]glutamate with the cofactor-independent glutamate racemase from *Lactobacillus*. This supports a deprotonation/protonation mechanism for racemization in which the breaking of the carbon–hydrogen bond at C-2 is partially rate-determining. Substantial "overshoots" were observed when the time course of racemization of either enantiomer of glutamate was monitored using circular dichroism spectroscopy. This is consistent with a "two-base" mechanism accompanied by a kinetic isotope effect. "Competitive deuterium washout" experiments were used to measure kinetic isotope effects on V_{max}/K_m of 2.5 for (*S*)-glutamate and 3.4 for (*R*)-glutamate. The ratio of the notably different isotope effects was confirmed by "double competitive deuterium washout" experiments. Site-directed mutagenesis was used to generate the mutant C73A and C184A enzymes. In each case the mutant enzymes were inactive as racemases. The two mutant enzymes are, however, capable of catalyzing the elimination of HCl from opposite enantiomers of *threo*-3-chloroglutamic acid, a process that presumably requires only one enzymic base. This finding indicates that the active sites of the mutant enzymes are intact and that the two cysteines flank the bound substrate molecule. It appears that cysteine-73 is responsible for the abstraction of the C-2 hydrogen from (*R*)-glutamate and cysteine-184 abstracts the proton from (*S*)-glutamate in the racemization reaction of the wild-type enzyme.

The previous paper outlines experiments designed to elucidate the number of enzymic bases responsible for the handling of substrate protons in the reaction catalyzed by glutamate racemase (Gallo et al., 1993). The observation that the product enantiomer contains exclusively solvent-derived isotope at the 2-position, whereas the recovered substrate enantiomer (after small extents of reaction) is devoid of isotopic label, is only consistent with a "two-base" mechanism. In this mechanism, one enzymic base abstracts the C-2 proton from one face of the substrate, and the conjugate acid of a second enzymic base delivers a proton to the opposite face, bringing about the inversion of configuration (Figure 1). The absence of label incorporation into recovered substrate also indicates that the enzymic bases are monoprotic, and several lines of evidence suggest cysteines as the catalytic residues (Gallo & Knowles, 1993). The two cysteine residues (cysteine-73 and cysteine-184) present in glutamate racemase therefore serve as attractive targets for site-directed mutagenesis studies. Implicit in the mechanism is the heterolytic cleavage of the carbon–hydrogen bond at C-2, and it seems likely that this elementary step will be at least partially rate-limiting. Thus, the racemization of [2-^{2}H]glutamate may be slowed by the existence of a primary kinetic isotope effect.

Another cofactor-independent racemase, proline racemase, has also been shown to employ a two-base mechanism in which the bases are provided by cysteine residues (Cardinale & Abeles, 1968). It was demonstrated in this case that the enzyme catalyzes the racemization of (*S*)-[2-^{2}H]proline 2.5 times more slowly than (*S*)-[2-^{1}H]proline. Furthermore, on following the full course of racemization of (*S*)-proline in D_2O polarimetrically, an "overshoot" was observed. The optical rotation of the solution, initially negative, rapidly approached zero, became transiently positive, and then finally returned to the equilibrium value of zero. This phenomenon was explained in terms of a two-base mechanism that results in the following sequence:

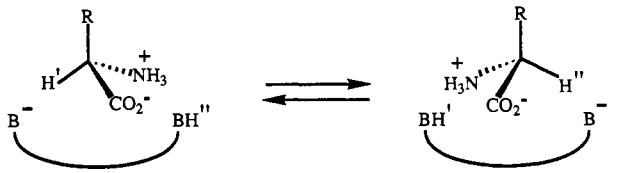

FIGURE 1: Two-base mechanism for enzyme-catalyzed amino acid racemization.

$$\underset{\text{proline}}{(S)\text{-}[2\text{-}^1\text{H}]} \rightarrow \underset{\text{proline}}{(R)\text{-}[2\text{-}^2\text{H}]} \rightleftarrows \underset{\text{proline}}{(S)\text{-}[2\text{-}^2\text{H}]}$$

When the optical rotation first reaches zero, equimolar amounts of (*S*)-proline and (*R*)-proline are present (isotopic substitution has a negligible effect on the specific rotation); however, the (*S*)-proline contains a mixture of protium and deuterium at the 2-position while the initial product (*R*)-proline is completely deuterated. Due to the kinetic isotope effect on 2-proton abstraction, the protium-containing molecules react more rapidly than the corresponding deuterium-containing species, resulting in a net flux toward the formation of the (*R*)-enantiomer, and an "overshoot" past an optical rotation of zero. Eventually, both enantiomers reach isotopic equilibrium (reflecting the isotopic content of the solvent), and the rotation returns to zero. Since Abeles' initial report, overshoots have been reported for mandelate racemase (Powers et al., 1991), 2-amino-ε-caprolactam racemase (Ahmed et al., 1986), and hydroxyproline 2-epimerase (Finlay & Adams, 1970).

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Further experiments designed to probe the isotopic dependency of the proline racemase reaction were later reported (Fisher et al., 1986a,b). A convenient and accurate method of determining V_{max}/K_m isotope effects was described and termed a "competitive deuterium washout" experiment. In this experiment an equimolar mixture of 2-deuterated substrate and unlabeled product in H_2O is treated with enzyme and as a consequence of the two-base mechanism, the label is "washed out" of the substrate into the solvent. The optical rotation of the solution, initially zero, is perturbed toward that of the substrate, reaches a maximum (or minimum, depending on which enantiomer is deuterated), and ultimately returns to zero. This perturbation is caused by the kinetic isotope effect that slows the reaction rate of the deuterated substrate relative to that of the protiated product. From the magnitude of the perturbation in the optical rotation, the value of the isotope effect on substrate racemization can be calculated. This technique is closely related to the equilibrium perturbation method described and analyzed by Cleland (Schimerlik et al., 1975; Cleland, 1977a).

In a related experiment, the "double competitive deuterium washout" experiment, a racemic mixture of the 2-deuterated amino acids is enzymatically equilibrated in H_2O. If the two enantiomers lose their label to solvent at different rates due to unequal kinetic isotope effects, a small transient perturbation in the optical rotation of the solution is observed. The magnitude of this perturbation can then be used to calculate the ratio of the kinetic isotope effects on the racemization of the two enantiomers (Fisher et al., 1986a).

In this paper, the V_{max} isotope effects on the racemization of (*R*)-[2-^{2}H]glutamate and (*S*)-[2-^{2}H]glutamate by glutamate racemase are reported. Overshoots are observed using circular dichroism spectroscopy and are in accord with a two-base mechanism accompanied by a primary deuterium isotope effect. Both competitive and double competitive deuterium washout experiments are reported and used to calculate the values of the V_{max}/K_m isotope effects in both directions. Site-directed mutagenesis experiments in which each of the cysteine residues of glutamate racemase is changed to an alanine are described, and each of these enzymes is used in a "probe reaction" which requires only one enzymic base, to confirm the active-site integrity of the mutants and to deduce which enzymic base deprotonates each enantiomer of glutamate.

EXPERIMENTAL PROCEDURES

Materials. Glutamate racemase was purified from *Escherichia coli* DH5α carrying the glutamate racemase expression vector pKG3 (Gallo & Knowles, 1993). Acylase I (grade II, porcine kidney) and glutamic–oxalacetic transaminase (type II-A, porcine heart) were purchased from Sigma (St. Louis, MO). Deuterium oxide (99.9 atom % excess) was purchased from Cambridge Isotope Laboratories (Woburn, MA). Ultrapure potassium phosphate (99.999%) and potassium hydroxide (99.99%) were purchased from Aldrich Chemicals (Milwaukee, WI). Ellipticity measurements were made on an Aviv 62DS CD spectrometer.

Synthesis of (R,S)-[2-^{2}H]Glutamic Acid. (*S*)-Glutamic acid (2.0 g, 13.6 mmol) was dissolved in D_2O (30 mL) and the pD was adjusted to 7.6 by the addition of NaOD (2 M). The mixture was freeze-dried, and dithiothreitol (10 mg) and D_2O (100 mL) were added. The solution was incubated at 35 °C, and glutamate racemase (105 units, in 250 μL of 50 mM triethanolamine hydrochloride buffer, pH 7) was then added. After 14 h, additional dithiothreitol (5 mg) and glutamate racemase (105 units, in 250 μL of 50 mM triethanolamine hydrochloride buffer, pH 7) were added. After a total of 38 h, the mixture was passed through a PM10 membrane in a stirred ultrafiltration cell (Amicon). The pD of the filtrate was adjusted to 3.22 with aqueous HCl (2 M), and the volume was reduced to approximately 10 mL under reduced pressure. Ethanol (25 mL) was added and the mixture was cooled at 4 °C for several hours. The resulting solids were collected by filtration and were then washed with ethanol and acetone and then dried under high vacuum. (*R,S*)-[2-^{2}H]Glutamic acid (1.65 g, 11.2 mmol) was obtained in 83% yield. ^{1}H NMR analysis[1] (500 MHz, D_2O) indicated that >98% of the product was deuterated at the 2-position, and circular dichroism spectroscopy at 204 nm in 10 mM sodium phosphate buffer, pH 8.0, indicated that complete racemization had occurred. This was confirmed by the derivatization of a small portion of the material with (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [as described in Gallo et al. (1993)] followed by GC/MS analysis.

Synthesis of N-Acetyl-(R,S)-[2-^{2}H]glutamic Acid. (*R,S*)-[2-^{2}H]Glutamic acid (1.47 g, 10 mmol) was dissolved in cold aqueous HCl (6 N, 3.4 mL) and placed in an ice bath. Acetic anhydride (1.5 mL, 16 mmol) was added, and the mixture was shaken with cooling until homogeneous (about 15 min). The mixture was then acidified to pH 1 with concentrated HCl and kept at 4 °C for several days. The resulting crystals were collected by filtration, washed with acetone, and then dried under high vacuum. *N*-Acetyl-(*R,S*)-[2-^{2}H]glutamic acid (1.14 g, 6.0 mmol) was obtained in a 60% yield. ^{1}H NMR analysis (500 MHz, DMSO-d_6) indicated that >98% of the product was deuterated at the 2-position.

Synthesis of (S)-[2-^{2}H]Glutamic Acid and N-Acetyl-(R)-[2-^{2}H]glutamic Acid. *N*-Acetyl-(*R,S*)-[2-^{2}H]glutamic acid (700 mg, 3.7 mmol) was dissolved in D_2O (25 mL), and the pD was adjusted to 7.5 with ammonium hydroxide. The solution was freeze-dried and the residue was dissolved in D_2O (30 mL). Acylase I (50 mg, 36 000 units) was added, and the mixture was incubated at 37 °C under argon. After 9 h, additional acylase I (25 mg, 18 000 units) was added, and the incubation was continued for a total of 18 h. Precipitated denaturated protein was removed by centrifugation, and the soluble protein was removed by ultrafiltration using a Centriprep-10 concentrator (Amicon, Beverly, MA). The pD of the resulting solution was adjusted to 3.22 with HCl (2 N), and the volume was then reduced to approximately 10 mL under reduced pressure. Ethanol (40 mL) was added and the mixture was cooled to 4 °C for several days. The resulting solids were collected by filtration, washed with ethanol, and then dried under high vacuum. (*S*)-[2-^{2}H]Glutamic acid (200 mg, 1.36 mmol) was obtained in a 73% yield. ^{1}H NMR analysis (500 MHz, D_2O) indicated that >98% of the product was deuterated at the 2-position and circular dichroism spectroscopy at 204 nm in 10 mM sodium phosphate buffer, pH 8.0, gave a molar ellipticity value of 30.8 ± 0.5 mdeg cm^{-1} mM^{-1} (in agreement with that of protio-(*S*)-glutamic acid: 31.0 ± 0.5 mdeg cm^{-1} mM^{-1}). A small portion of the material was derivatized with (*R*)-(+)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride [as described in Gallo et al. (1993)] and GC analysis indicated that <1% of the (*R*)-enantiomer was present. GCMS analysis of the derivative confirmed that >98% of the product was monodeuterated.

The filtrate from the ethanol precipitation step was evaporated to dryness under reduced pressure. The residue

[1] Abbreviations: NMR, nuclear magnetic resonance; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry.

was dissolved in H_2O (20 mL), and the solution was acidified to pH 1 with concentrated HCl and then evaporated to dryness under reduced pressure. The residue was redissolved in H_2O (5 mL) and cooled to 4 °C for several days. The resulting crystals were collected by filtration, washed once with cold water, followed by acetone, and dried under high vacuum. This produced *N*-acetyl-(*R*)-[2-^{2}H]glutamic acid (220 mg, 1.16 mmol) in 63% yield. ^{1}H NMR analysis (500 MHz, DMSO-d_6) indicated that >98% of the product was deuterated at the 2-position.

Synthesis of (R)-[2-^{2}H]Glutamic Acid. *N*-Acetyl-(*R*)-[2-^{2}H]glutamic acid (110 mg, 0.58 mmol) was dissolved in HCl (5 N, 5 mL), and the solution was heated at reflux (125 °C) for 4 h. The solution was evaporated to dryness under vacuum, and the residue was dissolved in H_2O (3 mL) containing triethylamine (1 mL). The mixture was evaporated to dryness under vacuum, the residue was dissolved in H_2O (1 mL), and ethanol (3 mL) was added. After several days at 4 °C, the resulting precipitate was collected by filtration, washed with ethanol, and then dried under high vacuum. (*R*)-[2-^{2}H]-Glutamic acid (41 mg, 0.28 mmol) was obtained in a 48% yield. ^{1}H NMR analysis (500 MHz, D_2O) indicated that >98% of the product was deuterated at the 2-position and circular dichroism spectroscopy at 204 nm in 10 mM sodium phosphate buffer, pH 8.0, gave a molar ellipticity value of -31.5 ± 0.5 mdeg cm^{-1} mM^{-1} (in agreement with that of protio-(*R*)-glutamic acid: -31.0 ± 0.5 mdeg cm^{-1} mM^{-1}). A small portion of the material was derivatized with *R*-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [as described in Gallo et al. (1993)], and GC analysis indicated that <1% of the (*S*)-enantiomer was present. GC/MS analysis of the derivative confirmed that >98% of the product was monodeuterated.

Measurement of V_{max} Isotope Effects for Glutamate Racemase. The initial velocity of the reaction catalyzed by glutamate racemase was measured at 30 °C by following the change in ellipticity at 204 nm with time, of a solution containing substrate (5 mM) ((*S*)-[2-^{2}H]glutamate, (*S*)-[2-^{1}H]glutamate, (*R*)-[2-^{2}H]glutamate, or (*R*)-[2-^{1}H]glutamate) in 10 mM potassium phosphate buffer, pH 8, containing dithiothreitol (0.2 mM). Data from the first 2–3% of each reaction were transferred to the graphics program KaleidaGraph for subsequent rate determination. The V_{max} isotope effect for each enantiomer was calculated as the ratio of the rate of reaction of unlabeled substrate to that of the labeled substrate of the same configuration.

Overshoot Experiments. Solutions containing (*R*)- or (*S*)-glutamic acid (3 mM) and potassium hydrogen phosphate (10 mM) were prepared in D_2O and adjusted to pH 8 with potassium hydroxide (2 N). The solutions were freeze-dried and redissolved in D_2O two times. To each glutamate/phosphate solution (2.94 mL) was added dithiothreitol (0.06 mL of 10 mM). After temperature equilibration at 30 °C, glutamate racemase (2 units) was added. The ellipticity at 204 nm was followed with time until it remained constant for several reaction half-lives. Control reactions were performed using glutamate racemase (0.7 units) and (*R*)- or (*S*)-glutamic acid (3 mM) in phosphate buffer (9.8 mM in H_2O), pH 8, containing dithiothreitol (0.2 mM) and were followed until the ellipticity at 204 nm remained constant. All data points were normalized by the subtraction of a "reference value" that was determined by measuring the ellipticity of a solution that was identical to the reaction solution except that it contained no substrate. The normalized data were transferred to the graphics program KaleidaGraph and plotted.

Competitive Deuterium Washout Experiments. A solution (2.995 mL) of equal concentrations of (*S*)-[2-^{2}H]glutamate and (*R*)-[2-^{1}H]glutamate (3 mM each) was prepared in 10 mM potassium phosphate buffer, pH 8, containing dithiothreitol (0.2 mM). After temperature equilibration at 30 °C, glutamate racemase (2 units) was added. The data were obtained and manipulated as above. The complementary experiment using a solution of equimolar (*R*)-[2-^{2}H]glutamate and (*S*)-[2-^{1}H]glutamate that was prepared as above was also performed.

Double Competitive Deuterium Washout Experiment. A solution (2.995 mL) containing racemic (*R*,*S*)-[2-^{2}H]-glutamate (3 mM) was prepared in 10 mM potassium phosphate buffer, pH 8, containing dithiothreitol (0.2 mM). After temperature equilibration at 30 °C, glutamate racemase (2 units) was added. The data were obtained and manipulated as above.

Site-Directed Mutagenesis and Preparation of Mutant Extracts. The recombination PCR procedure described by Jones and Winistorfer (1992) was used to perform the site-directed mutagenesis. Two PCR amplifications of the vector pKG3 (Gallo & Knowles, 1993) that had been linearized by digestion with *Pst*I were run: reaction 1 (C73A) contained primers 01 and 05 (see below for sequence) and reaction 2 (C184A) contained primers 03 and 05. Two PCR amplifications of vector that had been linearized by digestion with *Eco*RI were also run: reaction 3 (C73A) contained primers 02 and 06 and reaction 4 (C184A) contained primers 04 and 06. The combined products of reaction 1 with reaction 3, and of reaction 2 with reaction 4 were transformed directly into MAX Efficiency DH5α competent *E. coli* (GIBCO BRL, Gaithersburg, MD). Individual colonies that grew on LB plates containing ampicillin (100 μg/mL) were grown overnight at 37 °C in 10 mL of LB also containing ampicillin (100 μg/mL). Plasmid DNA was isolated from these cells using the Magic Minipreps DNA purification system (Promega, Madison, WI). The plasmids were denatured and sequenced in the area of the mutation using the modified T7 DNA polymerase, Sequenase (U.S. Biochemical Corp., Cleveland, OH), according to the manufacturer's protocols. Two mutant plasmids were chosen which contained the desired codon changes (C73A and C184A) and were transformed into MAX Efficiency DH5α competent *E. coli*. Two overnight cultures (7 mL each) were grown as described above. The cells were pelleted and then resuspended in 30 mM Trien–HCl buffer (1 mL), pH 7.5, containing (*R*,*S*)-glutamic acid (1 mM), glycerol (10% v/v), dithiothreitol (2 mM), pepstatin (1 mg/L), aprotinin (1 mg/L), and phenylmethanesulfonyl fluoride (1.5 mM, added immediately before cell lysis). The cells were lysed by passing them through a French pressure cell at 20 000 psi. Following ultracentrifugation at 40 000 rpm for 2 h to pellet the cell debris, the resulting supernatant was frozen in liquid nitrogen and stored at −75 °C.

Synthesis of Oligonucleotides. Oligonucleotides were synthesized on a MilliGen/Biosearch 7500 DNA synthesizer (MilliGen Corp., Bedford, MA) and purified using oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA). Concentrations were assessed from the absorbance at 260 nm. The following oligonucleotides (5′ to 3′) were synthesized:

01 TCGCCGTATTGGCGGCCACGACC

02 GGTCGTGGCCGCCAATACGGCGA

03 GGAAGTGGGTGGCGCCCATAATC

04 GATTATGGGCGCCACCCACTTCC

05 CGACGAGCGTGACACCACGATGCC

06 GCAGAGCGAGGTATGTAGGCGGTGC

where the C73A (primers 01 and 02) and the C184A (primers 03 and 04) codons are underlined. Primers 05 and 06 are complementary to the pUC18 vector.

Synthesis of Racemic threo-3-Chloroglutamate. Racemic *threo*-3-chloroglutamate was synthesized by the procedure of Khomutov et al. (1966) with slight modifications in the final step. Diethyl *threo*-3-chloroglutamate hydrochloride (360 mg, 1.5 mmol) was dissolved in 20% HCl (10 mL) and heated in a boiling water bath for 1 h. The solution was evaporated to dryness under reduced pressure, and the residue was redissolved in 10 mM potassium phosphate buffer (20 mL, pH 7). This solution was applied to a column (15 mL) of AG1-X8 (formate form, 100–200 mesh) (Bio-Rad Laboratories, Richmond, CA). The column was washed with H_2O (40 mL) and eluted with formic acid (0.25 N, 80 mL). The fractions containing product (as indicated by ninhydrin staining) were evaporated to dryness under reduced pressure. Racemic *threo*-3-chloroglutamate (140 mg, 0.77 mmol) was obtained in a 51% yield.

Conversion of threo-3-Chloroglutamate to α-Ketoglutarate by Wild-Type Racemase. A solution of racemic *threo*-3-chloroglutamic acid (30 mM, 1 mL) in D_2O was added to deuterated 100 mM potassium phosphate buffer (1 mL), pD 8, containing dithiothreitol (0.5 mM), and the mixture was divided into two NMR tubes (final pD 7, by pH paper). To one of the tubes was added glutamate racemase in the same deuterated potassium phosphate buffer (7 units in 50 μL). The tubes were incubated at 37 °C, and the progress of the reaction was monitored using ^{1}H NMR spectroscopy (500 MHz). A control sample of α-ketoglutarate was prepared and monitored in an identical fashion.

Treatment of threo-3-Chloroglutamate with Mutant Extracts. The extracts containing the mutant enzymes described earlier were thawed and concentrated to 100 μL at 5 °C using an ultrafiltration membrane [10 000-MW cutoff, Centricon-10 concentrators (Amicon, Beverly MA)]. To each of the samples was added deuterated 100 mM potassium phosphate buffer (1 mL), pD 8, containing 0.25 mM dithiothreitol, and the samples were concentrated to 100 μL. A second addition of buffer (1 mL), followed by concentration to 200 μL, prepared the extracts for use. A solution of racemic *threo*-3-chloroglutamate (20 mM, 2 mL) in the same buffer was prepared and divided into two NMR tubes. To one of the tubes was added an aliquot (60 μL) of extract containing the C73A mutant and to the other was added an aliquot (60 μL) of extract containing the C184A mutant. The tubes were incubated at 37 °C, and the reactions were monitored using ^{1}H NMR. After 2 h, a second aliquot (60 μL) was added to each tube. After 3 h, 300 μL was removed from each tube, added to 200 μL of the deuterated buffer in a separate NMR tube, and treated with glutamic–oxalacetic transaminase (100 units in 30 μL of the deuterated buffer). To the remaining 800 μL of each reaction was added an aliquot of the extract containing the mutant that was not added initially. These reactions were monitored for a total of 5 h.

RESULTS AND DISCUSSION

Direct Measurement of V_{max} Isotope Effects. Racemic [2-^{2}H]glutamate was prepared by the enzyme-catalyzed racemization of (*S*)-glutamate in D_2O.[2] Following chemical N-acylation, the isotopically-labeled substrates were resolved using acylase I, which accepts only the (*S*)-enantiomer (Manesis & Goodman, 1987).[3] Recrystallization of the remaining material, followed by acid hydrolysis of the *N*-acetyl group, afforded the isotopically-labeled (*R*)-enantiomer.

Using circular dichroism spectroscopy, the initial rates of the enzyme-catalyzed racemization of the all-protio and of the monodeutero enantiomers of glutamic acid were measured under saturating conditions. With either enantiomer as substrate, the racemization of the 2-deuterated amino acid was found to be slowed by a substantial primary kinetic isotope effect. For the racemization of (*S*)-glutamate, $^HV_{max}/^DV_{max} = 2.2 \pm 0.4$, and for (*R*)-glutamate, $^HV_{max}/^DV_{max} = 3.1 \pm 0.5$. The rather large uncertainty in these values is due to the difficulties in following small extents of reaction using a relatively insensitive assay.

The simplest kinetic scheme that describes the racemase reaction under saturation conditions is

$$E_1 + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \xrightarrow{k_3} E_2 + P$$

In the above scheme, interconversion of the two forms of the free enzyme, E_1 and E_2, which (by analogy with proline racemase) are presumed to differ only in the protonation state of the two enzymic bases, is assumed to be rapid compared with the turnover of substrate (Gallo et al., 1993). Using Cleland's method of net rate constants (Cleland, 1975), the maximal velocity of the racemase reaction can be expressed as

$$V_{max} = E_t k_2 k_3/(k_2 + k_{-2} + k_3)$$

where E_t is the total concentration of enzyme. If k_3 is very large compared with k_2 and k_{-2} (that is, if substrate dissociation is much faster than the chemical steps of the reaction), the V_{max} isotope effect reduces to the intrinsic isotope effect, $^Hk_2/^Dk_2$. The two-base mechanism for racemization predicts that k_2 is the rate constant for the deprotonation/reprotonation of glutamate at C-2 (in either a concerted or a stepwise reaction). The existence of a primary isotope effect is therefore fully consistent with the proposed carbon–hydrogen bond breaking process.

Overshoot Experiments. The observation that the product of the racemase reaction contains solvent-derived isotope at the 2-position whereas the recovered substrate does not, and the finding that there is a primary isotope effect on the rate of racemization, implies that the racemase reaction will exhibit overshoot patterns when the reaction of unlabeled substrates is run in D_2O. Panels A and B in Figure 2 show the traces of ellipticity versus time for the racemization of solutions of (*R*)- and (*S*)-glutamate in H_2O, respectively. Panels C and D show the corresponding traces when the reaction is run in D_2O. With either enantiomer serving as substrate, a sub-

[2] When the racemization was carried out using the nonenzymatic conditions reported by Manesis and Goodman (1987), a small amount (5–10%) of dideuteroglutamate was detected by GC/MS analysis. We believe this material is 2,4-dideuteroglutamic acid, and for this reason (*R,S*)-[2-^{2}H]glutamic acid was prepared by an enzymatic racemization procedure.

[3] When using the commercially available acylase I in H_2O, the product (*S*)-glutamic acid was found to be enantiomerically pure, but some loss (5–15%) of the 2-deuterium label was observed. This loss may derive from a transaminase contaminant that catalyzes a stereospecific proton exchange. For this reason, the deacylation was performed in D_2O, and the product was found to be both enantiomerically and isotopically pure.

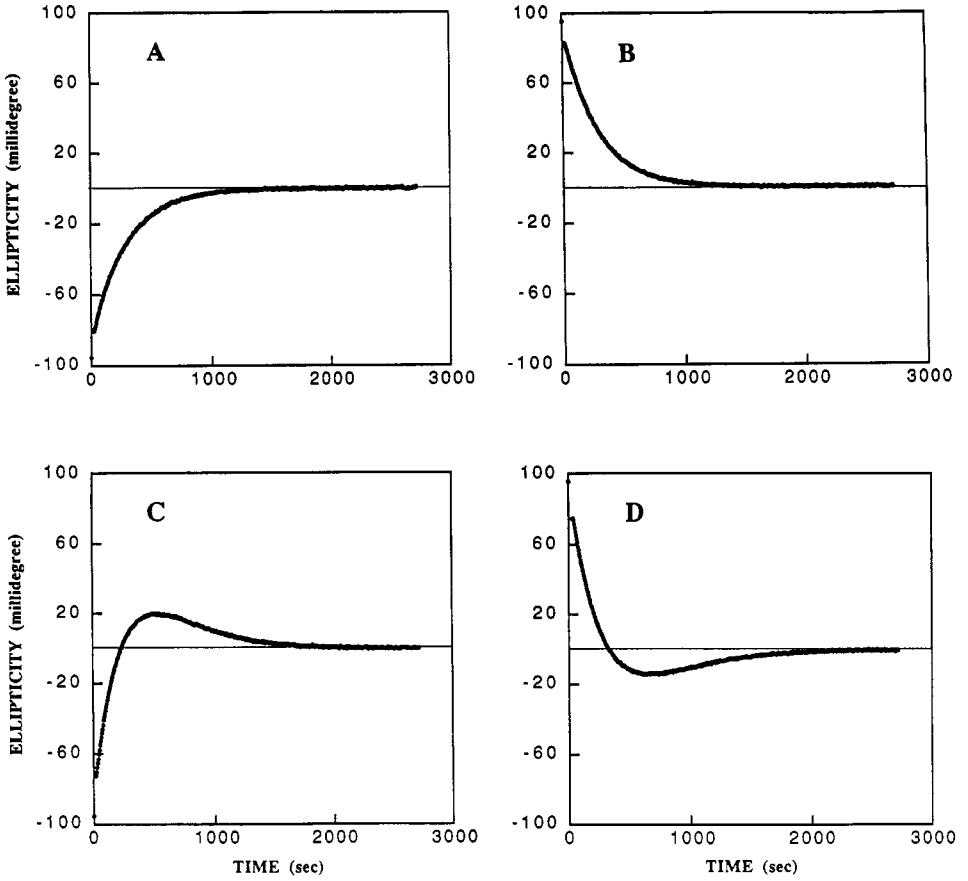


FIGURE 2: Ellipticity at 204 nm for the racemization of glutamate enantiomers: (A) (*R*)-glutamate in H_2O; (B) (*S*)-glutamate in H_2O; (C) (*R*)-glutamate in D_2O; (D) (*S*)-glutamate in D_2O.

stantial overshoot past a value of zero ellipticity is observed, followed by a return to the equilibrium value of zero.

The observation of "overshoots" with each substrate is the consequence of a two-base mechanism in which deuterium is incorporated solely into the product enantiomer, coupled with the existence of a primary isotope effect. It is interesting to consider whether an enzyme that operates via a one-base mechanism could display "overshoot" phenomena. In a case where the same enzymic base is responsible for both proton abstraction and reprotonation, solvent-derived isotope can be incorporated into the substrate or into the product, or both, *either* if the labilized proton exchanges with bulk solvent during the lifetime of the deprotonated substrate intermediate *or* if the enzymic base is polyprotic. Furthermore, if the solvent isotope partitions unequally between the substrate and product enantiomers, an isotopic imbalance between substrate and product pools could develop, resulting in the observation of an overshoot. However, since the enzyme–intermediate complexes formed in the forward and in the reverse reactions must be identical, the partitioning of the solvent isotope must also be the same. Therefore, the observation of an overshoot in one reaction direction for an enzyme that operates via a one-base mechanism should preclude an overshoot in the reverse direction. This logic is at odds with the reports on the mechanism of the pyridoxal phosphate-dependent enzyme, 2-amino-ε-caprolactam racemase, for which overshoots were observed in both reaction directions, even though a single base is thought to be involved in proton abstraction (Ahmed et al., 1986). In that work, the magnitude of the observed overshoot when the (*R*)-enantiomer served as substrate was, however, very small. Furthermore, the one-base mechanism was deduced from NMR measurements, which are much less sensitive than the mass spectroscopic methods usually employed.

Interestingly, the magnitude of the overshoot that we observe in the racemization of (*R*)-glutamate differs from that seen with (*S*)-glutamate. When (*S*)-glutamate is the substrate, the ellipticity overshoots zero by about 16% of the initial ellipticity, whereas with (*R*)-glutamate as the starting substrate, the overshoot (in the opposite direction) amounts to about 22% of the starting value. Cleland has shown that the magnitude of the overshoot is related to the size of the V_{max}/K_m isotope effect for a two-base racemase (Cleland, 1977b). It thus appears that the kinetic isotope effect is larger with (*R*)-glutamate as substrate, which is consistent with the directly measured V_{max} isotope effects. To obtain more accurate values of the V_{max}/K_m isotope effects (in H_2O instead of D_2O), competitive deuterium washout experiments were performed.

Competitive Deuterium Washout Experiments. In competitive deuterium washout experiments, equimolar mixtures of 2-deuterated substrate and its 2-protio enantiomer are treated with racemase, and the deuterium label is "washed out" into bulk solvent. Figure 3A shows the time course of the ellipticity for the deuterium washout from (*R*)-[2-^{2}H]-glutamate, and Figure 3B shows the analogous trace for (*S*)-[2-^{2}H]glutamate. In both cases, the initial ellipticity is zero, since isotopic substitution does not significantly alter the molar ellipticity of glutamate. Following the addition of the racemase, the ellipticity is perturbed toward that of the labeled enantiomer. The ellipticity eventually returns to zero when all of the label has been washed out into solvent and a racemic mixture of protioglutamate remains.

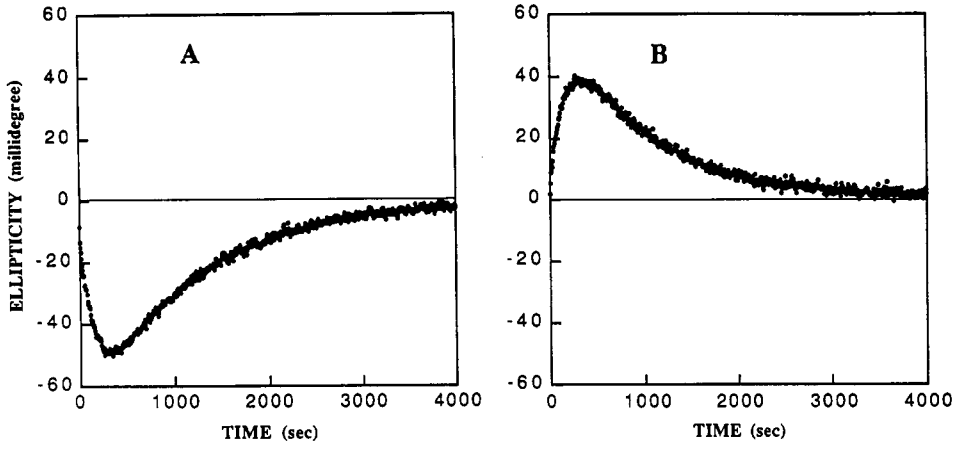


FIGURE 3: Ellipticity at 204 nm in the competitive deuterium washout experiments: (A) (*R*)-[2-^{2}H]glutamate and (*S*)-glutamate; (B) (*S*)-[2-^{2}H]glutamate and (*R*)-glutamate.

A mathematical treatment of the washout experiment can be found in Fisher et al. (1986a) which describes studies on the energetics of proline racemase. Proline racemase also employs a cofactor-independent, two-base mechanism and the equations are relevant to the present experiment. Using "composite" rate constants, eq 1 relates the size of the maximum perturbation, λ_{max} (expressed as a mole fraction of total glutamate), to the V_{max}/K_m isotope effect, ${}^{H}k_{1,2,3}/{}^{D}k_{1,2,3}$:

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

where

$$R = 2{}^{H}k_{1,2,3}/{}^{D}k_{1,2,3}$$

Applying eq 1 to the data shown in Figure 3A, where λ_{max} = 0.21, an isotope effect of 2.5 ± 0.1 is calculated for the racemization of (*S*)-glutamate. For the racemization of (*R*)-glutamate (Figure 3B), the measured λ_{max} = 0.26, giving an isotope effect of 3.4 ± 0.1. These data show that there is indeed a substantial difference in the isotope effects for the reaction of the two enantiomeric substrates. This is possible because even though the substrate and product are enantiomeric, the enzyme–substrate and enzyme–product complexes are not. Furthermore, this difference in isotope effects does not require that the two catalytic bases be chemically different. For example, proline racemase, which is thought to employ two thiol groups as bases, one from each of two identical subunits, also exhibits a (somewhat smaller) difference in isotope effects for its two substrates (Fisher et al., 1986a,b).

Double Competitive Deuterium Washout Experiment. The double competitive deuterium washout experiment provides a sensitive method to measure the *ratio* of the isotope effects in the two directions. In this experiment, an equimolar mixture of *both* 2-deuterated enantiomers is treated with the racemase, and the two substrates compete in a race to "wash out" their isotopic label. If the isotope effects are unequal, a net flux of material will result in the direction of the enantiomer with the larger isotope effect. The magnitude of this perturbation is dependent on the ratio of the two isotope effects. The result for the glutamate racemase reaction is shown in Figure 4, which clearly illustrates that a perturbation in the ellipticity of the solution (initially at zero for a racemic mixture) toward that of (*R*)-glutamate has occurred. This demonstrates that the (*R*)-enantiomer has the larger V_{max}/K_m isotope effect and is in good agreement with the previous results.

Data from the double competitive deuterium washout experiment yield the ratio of the isotope effects, using the

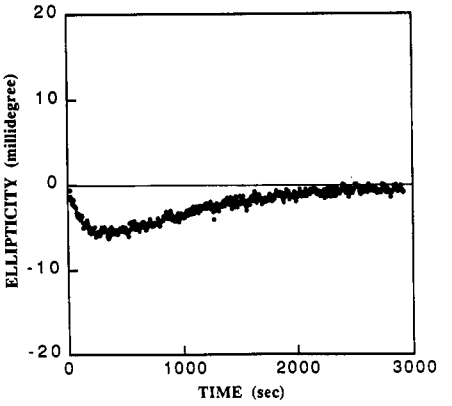


FIGURE 4: Ellipticity at 204 nm in the double competitive deuterium washout experiment using (*R*,*S*)-[2-^{2}H]glutamate.

derivation of Fisher et al. (1986a):[4]

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

where ξ is the ratio of the V_{max}/K_m isotope effects. Treatment of the results obtained for the racemization of glutamate, where λ_{max} = 0.058, gives a ratio of the isotope effect for (*S*)-glutamate to that for (*R*)-glutamate of 0.73. This value is in excellent agreement with the independently-measured isotope effects in the competitive deuterium washout experiments, which give a ratio of 0.74.

Properties of Cysteine-to-Alanine Mutants of Glutamate Racemase. The proposal that the two cysteine residues of glutamate racemase serve as the enzymic bases in the racemization reaction was investigated by independently replacing each with an alanine residue. The method of recombination PCR (Jones & Winistorfer, 1991; Jones & Howard, 1991) was used to perform site-directed mutagenesis on the glutamate racemase expression vector pKG3 (Gallo & Knowles, 1993). Both mutant enzymes, C73A and C184A, were overproduced in *E. coli*, and polyacrylamide gel electrophoresis of the cell-free extracts under denaturing conditions

[4] The factor of 2 in eq 2 derives from the fact that equimolar amounts of the deuterated enantiomers are present at the start of this experiment. The analogous equation, found in Fisher et al. (1986a) (eq 32, in that paper), contains a factor of 4 since the starting conditions used by these workers included equimolar amounts of the unlabeled enantiomers as well. The unlabeled species were omitted in this work to maximize the sensitivity. The experiment is still under "clamped" conditions in the sense that there are essentially equimolar amounts of the two enantiomers present throughout the experiment.

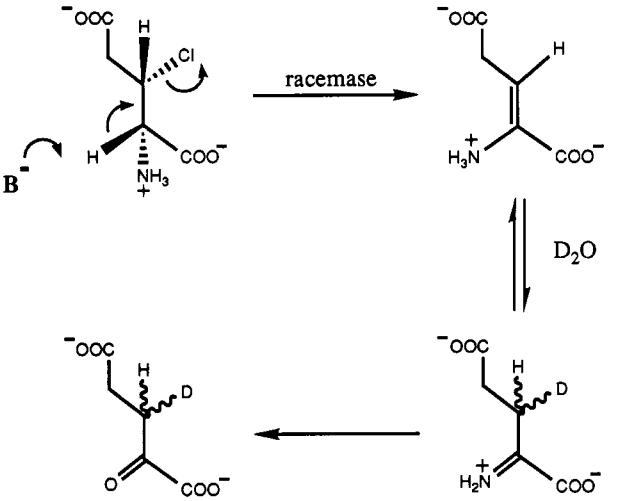
showed a prominent band which comigrated with the wild-type racemase. These extracts were found to be devoid of any detectable glutamate racemase activity using the end-point assay described earlier (Gallo & Knowles, 1993), which suggests that a catalytically critical residue has indeed been removed in each case.

Enzymatic Conversion of threo-3-Chloroglutamate to 2-Ketoglutarate. To study mutants of glutamate racemase in which one of the two active-site bases has been removed, a "probe reaction" was employed. This reaction is the elimination of HCl from *threo*-3-chloroglutamate, and it is mechanistically reasonable to suggest that this process would require only one of the two enzymic bases. This type of experiment has nicely illuminated the mechanism of the "two-base" enzyme, mandelate racemase. In this case, replacement of an active site histidine with an asparagine produces a mutant that has no racemase activity but is capable of eliminating HBr exclusively from the (*S*)-enantiomer of *p*-(bromomethyl)-mandelate, converting it into *p*-(methyl)benzoylformate (Landro et al.,1991). Since the wild-type mandelate racemase is able to handle both enantiomers of the brominated substrate analogue, the authors concluded that the histidine moiety is responsible for deprotonation of the (*S*)-enantiomer.

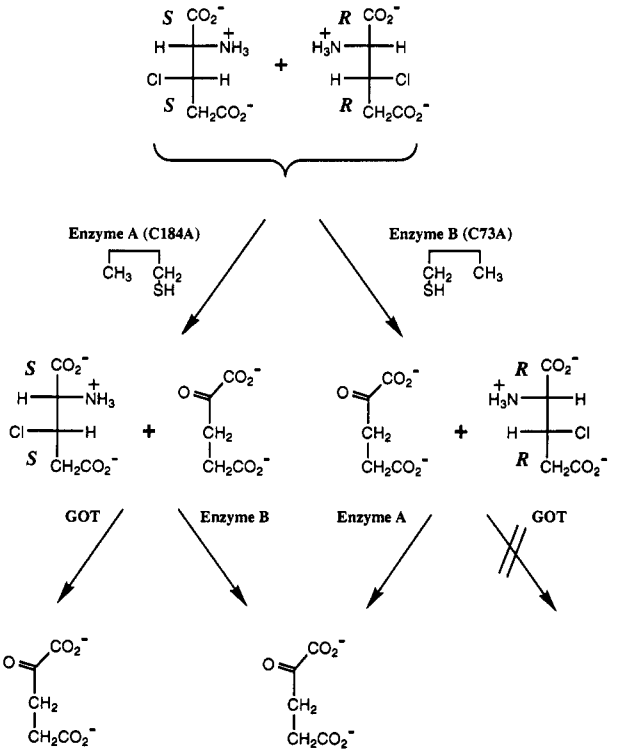
In the present case, when racemic *threo*-3-chloroglutamate is treated with wild-type glutamate racemase in D_2O and the reaction progress is monitored by ^1H NMR, the resulting spectra show that each of the enantiomers of chloroglutamate is enzymatically converted to 2-ketoglutarate which is initially monodeuterated at C-3. This conversion occurs at a rate that is about an order of magnitude slower than the enzyme catalyzed incorporation of solvent deuterium into glutamic acid itself (Gallo et al., 1993). Gradually, the 2-ketoglutarate produced becomes dideuterated at C-3 as a result of the nonenzyme-catalyzed exchange of these protons with solvent. When an authentic sample of 2-ketoglutarate is incubated in deuterated buffer in the absence of the racemase, signals identical to those seen in the racemase reaction are observed, as the C-3 protons of the 2-ketoglutarate slowly exchange with solvent deuterium (the half-life for exchange is approximately 15 h). Control reactions of chloroglutamate containing no enzyme show no detectable conversion to 2-ketoglutarate under identical conditions, indicating that the process is indeed catalyzed by the racemase.

The observation of the glutamate racemase-catalyzed conversion of *threo*-3-chloroglutamate to 2-ketoglutarate supports a deprotonation/reprotonation mechanism for the racemization of glutamate. It is likely that the enzyme first promotes the elimination of HCl to give the enamine shown in Scheme I. Free in solution, this compound rearranges to the imine, incorporating solvent deuterium at the 3-position. The resulting imine then spontaneously hydrolyzes to 2-ketoglutarate and ammonia. It is interesting to note that no evidence for the formation of the erythro diastereomers is seen, indicating that the elimination of HCl is a more rapid process than epimerization at C-2, with β-chlorinated substrates. The conversion of one of the enantiomers of *threo*-3-chloroglutamate into 2-ketoglutarate has also been reported to be catalyzed by glutamic–oxalacetic transaminase, an enzyme that utilizes pyridoxal phosphate as the cofactor in activating the 2-position of its substrates, and a similar reaction sequence was proposed (Manning et al., 1968). The observation that (*R*)-glutamate and (*R*)-aspartate do not act either as substrates or as inhibitors of glutamic–oxalacetic transaminase (Jenkins et al., 1959) suggests that this enzyme reacts only with the (2*S*,3*S*)-3-chloroglutamate enantiomer.

Scheme I: Enzymatic Conversion of *threo*-3-Chloroglutamate into 2-Keto[3-^{2}H]glutarate



Scheme II: Treatment of Racemic *threo*-3-Chloroglutamate with Glutamate Racemase Mutants[a]



[a] GOT is glutamic–oxalacetic transaminase.

Analysis of Mutant Enzymes with threo-3-Chloroglutamate. The "probe reaction" described above can be used in studies with the mutant enzymes in order to establish two important results. First, we show that the loss of racemase activity in the mutants is due to the absence of a catalytic base and not to a conformational change in the active site. Second, we can assign which enzymic base is responsible for the deprotonation of a given glutamate enantiomer. The experiment that was performed is outlined in Scheme II. To half of a sample of racemic *threo*-3-chloroglutamate was added cell-free extract rich in the mutant enzyme A (C184A), and to the other half was added an extract rich in the mutant enzyme B (C73A). The progress of the resulting reactions was then monitored using ^1H NMR, and it was found that in each case, half of the chloroglutamate was converted to 2-ketoglutarate. Addition of fresh extract to each of the

samples resulted in no further reaction,[5] indicating that inactivation of the enzyme was not responsible for the halted reaction. When a portion of the sample treated with enzyme A was incubated with glutamic–oxalacetic transaminase, the complete conversion to 2-ketoglutarate was observed, whereas with the sample that had been treated with enzyme B, no further reaction occurred. Since glutamic–oxalacetic transaminase reacts with only one enantiomer of *threo*-3-chloroglutamate [presumably that having the (*S*)-configuration at C-2], it appears that enzyme A handles only (2*R*,3*R*)-3-chloroglutamate and enzyme B handles only the (2*S*,3*S*)-enantiomer. To test this notion further, a portion of the enzyme A-treated sample was incubated with enzyme B, and vice versa. In both cases the complete conversion of the 3-chloroglutamate into 2-ketoglutarate occurred.

The observation that each of the mutant enzymes is capable of catalyzing an elimination reaction on a substrate analogue supports the notion that the mutagenesis has not greatly affected the structure of the active site. Furthermore, each mutant is capable of handling only one enantiomer of the chloroglutamate, suggesting that the two cysteine residues lie on opposite sides of the C-2 carbon. Since both mutants are incapable of racemizing glutamate, it is reasonable to assume that cysteine-73 and cysteine-184 provide the two catalytic bases necessary for the racemization of glutamate by the wild-type enzyme. The observation that enzyme A is capable of handling only (2*R*,3*R*)-3-chloroglutamate suggests that cysteine-184 is responsible for the abstraction of the C-2 hydrogen from (*S*)-glutamate in the wild-type racemization and that cysteine-73 abstracts the hydrogen from (*R*)-glutamate.

MECHANISTIC CONCLUSIONS

The cofactor-independent amino acid racemases provide an intriguing area of study for the mechanistic enzymologist. They belong to a varied class of enzymes that use active site bases to abstract a proton from a carbon acid. The difference in pK_a values of a protonated active site base and the substrate's carbon-bound proton seems to preclude the formation of significant levels of a discrete carbanionic intermediate (Thibblin & Jencks, 1979). This problem was addressed in studies on thiolase I from porcine heart, which demonstrated that the rate-determining step in the condensation between an acylenzyme intermediate and acetylcoenzyme A occurred after proton transfer from carbon (Gilbert, 1981). The issue has recently been analyzed in detail by Gerlt and his collaborators (Gerlt et al., 1991; Gerlt & Gassman, 1992), who have concluded that enzymatic reactions involving proton abstraction from carbon acids necessarily involve general acid catalysis. In the case of proton abstraction from α-amino acids, this would predict the formation of the monoanion of a geminal enediol which interacts with a cationic center at the active site.

In this series of papers, we have reported mechanistic studies on glutamate racemase. This enzyme has been shown to be devoid of carbonyl-containing cofactors such as pyridoxal phosphate or a pyruvoyl group, and it is unaffected by metal-chelating reagents (Gallo & Knowles, 1993). There is no evidence for the formation of an acylenzyme intermediate (Gallo et al., 1993). It thus appears likely that the enzyme simply employs general acid–base catalysis in effecting the racemization of glutamic acid.

[5] A slow background rate of conversion of (2*S*,3*S*)-3-chloroglutamate to 2-ketoglutarate was observed. This was attributed to the presence of transaminases in the cell extracts and accounted for <5% of the total conversion under these reaction conditions.

Glutamate racemase employs two enzymic bases in the racemization reaction (Gallo et al., 1993). One base deprotonates the substrate, and the conjugate acid of the other base reprotonates the substrate from the opposite face. The two-base mechanism has been inferred from the observations that solvent isotope is efficiently incorporated into the product enantiomer but not into the substrate enantiomer, irrespective of which enantiomer serves as substrate. The mutants C73A and C184A are both inactive with respect to the racemization of glutamate, yet each is capable of promoting the elimination of HCl from one of the enantiomers of *threo*-3-chloroglutamate (a process presumably requiring only one enzymic base). This indicates that cysteine residues are the enzymic bases that flank the C-2 position of enzyme-bound glutamate.

The isotope effect studies reported in the present paper further support the proposed mechanism. Primary deuterium isotope effects on V_{max} were directly measured for the racemization of each enantiomer. The carbon–hydrogen bond at C-2 is broken in an elementary step that is at least partially rate-determining and is consistent with a deprotonation/reprotonation mechanism. If the reaction is concerted, there is only one elementary step that does not involve binding, and this step is, of course, isotopically sensitive. If the reaction occurs in a stepwise fashion, the isotope effects reflect a composite of two partially rate-limiting transition states, one involving the formation of an intermediate and the other involving its collapse.

The competitive deuterium washout experiments provide an accurate and convenient method for measuring the V_{max}/K_m isotope effects for the racemase-catalyzed reaction. In these experiments, the magnitude of the isotope effect measured is independent of the enzyme concentration and does not rely on the accurate measurement of initial rates, thus eliminating many possible sources of error. The substantial V_{max}/K_m isotope effects measured in both reaction directions indicate that the binding steps are not rate-determining. A noticeable inequality between the isotope effects has been observed, and the ratio of these values is confirmed in the double competitive deuterium washout experiment. It is interesting to compare these results to those obtained with proline racemase, a dimeric enzyme that utilizes two identical cysteine residues, one from each subunit, during racemization. The smaller difference between the isotope effects that has been measured for proline racemase (Fisher et al., 1986a) may be explained by the necessarily similar environment that these bases occupy, as compared to the cysteines of glutamate racemase, both of which reside on a single polypeptide chain.

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