Mechanism of the Reaction Catalyzed by Glutamate Racemase†

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ABSTRACT: The mechanism of the cofactor-independent glutamate racemase from *Lactobacillus* has been studied. The possible formation of an acylenzyme intermediate during catalysis has been investigated using ^{18}O-carboxyl labeled glutamate. The absence of any washout of label during racemization argues against intermediate formation. The observation of the enzyme-catalyzed incorporation of deuterium at the C-2 position of glutamate upon racemization in D_2O provides evidence for a deprotonation/protonation mechanism. Further experiments have been performed in order to determine the number of enzymic bases responsible for racemization. Solvent deuterium is efficiently incorporated into the product enantiomer but not into the recovered substrate enantiomer in each reaction direction. This finding is consistent with a "two-base" mechanism in which one enzymic base deprotonates the substrate, and the conjugate acid of a second enzymic base protonates the resulting intermediate from the opposite face. It also suggests that the two bases are monoprotic. The possibility that the two enzymic forms, which differ at the very least by the protonation states of the active-site bases, are kinetically significant has been examined by measuring the entire time course of the the approach to equilibrium at various concentrations of glutamate. An "oversaturated" regime [Fisher, L. M., Albery, W. J., & Knowles, J. R. (1986) *Biochemistry 25,* 2529–2537] was not observed using glutamate concentrations as high as 100 mM, indicating that the two enzyme forms are rapidly interconverting under physiological conditions.

The first paper in this series describes the purification, cloning, overexpression, and initial characterization of glutamate racemase from *Lactobacillus fermenti* (Gallo & Knowles, 1993). The enzyme appears to catalyze the interconversion of the enantiomers of glutamic acid without the use of any cofactors, metal ions, or cosubstrates. It seems that the racemase relies completely on acid–base catalysis in the racemization process.

Initial studies on glutamate racemase from *Pediococcus pentosaceus* demonstrated that solvent-derived deuterium is incorporated into the 2-position of glutamic acid during the course of the enzyme-catalyzed racemization in D_2O (Nakajima et al., 1988). Solvent isotope incorporation at the stereogenic center argues strongly for a mechanism that proceeds by a deprotonation/reprotonation pathway, as distinct from either oxidation/reduction or elimination/addition pathways. Since the enzymic reaction shows no requirement for a cofactor (such as pyridoxal phosphate), the question remains as to how the enzyme labilizes the nonacidic proton at carbon-2 of the substrate.

The closest mechanistic precedent for the glutamate racemase reaction is found in studies on proline racemase, which is the paradigmatic cofactor-independent racemase. This enzyme incorporates solvent isotope into the 2-position of the substrate during racemization, as has been shown by the pioneering work of Cardinale and Abeles (1968). In this paper, the distinction was made between a "one-base" and a "two-base" mechanism. These mechanisms were first described by Rose (1966) to categorize enzymes that catalyze proton transfers. In the "two-base" racemase mechanism (Figure 1A), one enzymic base removes the C-2 proton from the substrate,[1] and the conjugate acid of a second enzymic base delivers a proton to the opposite face. In a "one-base" mechanism (Figure 1B), a single enzymic base serves to both deprotonate and reprotonate the amino acid. By showing that the rate of deuterium incorporation into the total pool of proline is equal to the rate of product formation during the initial phase of the reaction, Cardinale and Abeles (1968) deduced that proline racemase operates via a two-base mechanism. Two-base mechanisms have been reported for several enzymes, including diaminopimelic acid epimerase (Wiseman & Nichols, 1984), hydroxyproline 2-epimerase (Finlay & Adams, 1970), methylmalonylCoA epimerase (Leadlay & Fuller, 1983; Fuller & Leadlay, 1983), ribulose-5-phosphate epimerase (Davis et al., 1972), and mandelate racemase (Powers et al., 1991). A one-base mechanism has been proposed for 2-amino-ϵ-caprolactam racemase (Ahmed et al., 1986), for several alanine racemases (Faraci & Walsh, 1988), and for the nonspecific amino acid racemase of *Pseudomonas striata* (Shen et al., 1983).

Further investigations into the mechanism of proline racemase proved that there are actually two forms of the free enzyme, one that binds (*S*)-proline and one that binds (*R*)-proline (Rudnick & Abeles, 1975). These forms are thought to differ only in the protonation state of the two enzymic bases, as indicated in Figure 2. Under certain conditions the two forms were found to be kinetically significant (Fisher et al., 1986), and these observations led to the description of a kinetic regime known as "oversaturation". At high concentrations of proline, the net rate of the enzyme-catalyzed reaction (under reversible conditions) decreases with increasing substrate concentration. This results from the competition

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[1] The postulated intermediates shown in Figure 1 are protrayed as carbanions; however, it has recently been suggested by Gerlt (Gerlt et al., 1991; Gerlt & Gassman, 1992) that deprotonations of this type proceed directly through the enol tautomer of the acid, via a concerted protonation at the carbonyl oxygen.

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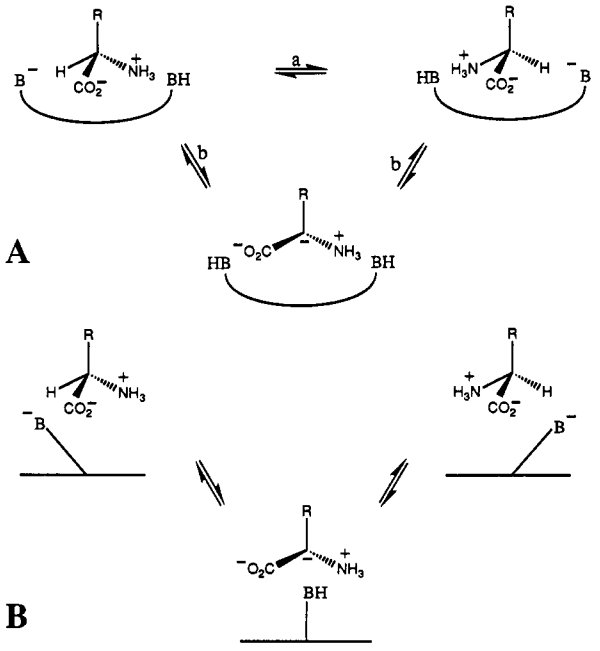

FIGURE 1: Mechanisms for amino acid racemases: (A) using two bases (a) concerted, (b) stepwise; (B) using one base.

between the two modes of regenerating the substrate-handling enzyme form: either via reconversion of product back into substrate (E_2 going to E_1 via the upper pathway in Figure 2) or by the direct interconversion of the two enzyme forms (lower pathway in Figure 2). When an oversaturating concentration of proline is subjected to the action of proline racemase, the amount of product formed rapidly reaches the point where the nonproductive upper pathway is followed a significant fraction of the time, and the approach to equilibrium is actually slowed.

In this paper, the possibility of acylenzyme intermediate formation during the enzyme-catalyzed racemization of glutamate has been examined by testing for the washout of ^{18}O isotope from carboxyl-labeled glutamic acid. Deuteration at the C-2 position of glutamate during racemization by the *Lactobacillus* enzyme in D_2O is reported, and the number of enzymatic bases required for the deprotonation/reprotonation events has been elucidated using solvent isotope incorporation studies. Further, the possibility of an oversaturation regime in the glutamate racemase reaction has been investigated.

EXPERIMENTAL PROCEDURES

Materials. Glutamate racemase was purified from *Escherichia coli* DH5α carrying the glutamate racemase expression vector pKG3 (Gallo & Knowles, 1993). (*R*)-(+)-MTPA[2] was purchased from Aldrich and converted to MTPA-Cl by the method of Dale et al. (1969). Deuterium oxide (99.9 atom % excess) was purchased from Cambridge Isotope Laboratories. Ellipticity measurements were made on an Aviv 62DS CD spectrometer.

Preparation and Racemization of [^{18}O]Glutamate. [^{18}O]-H_2O (95+ atom % excess, E G & G Mound Applied Technologies, Miamisburg, OH) (200 μL) was added to solid (*S*)-glutamate (20 mg) in a 0.3-mL Reacti-Vial (Pierce, Rockford, IL). A fine stream of HCl gas was bubbled through the solution until the glutamate dissolved (approximately 30 s), at which time the pH was approximately 1 (as determined by pH paper). The solution was then heated in the sealed vial (fitted with a Teflon cap) for 4 days in a heat block at 90 °C. A portion of the solution (44 μL) was diluted into phosphate buffer (10 mM, 6 mL). The final pH of the solution was 8. Dithiothreitol (120 μL of a 10 mM solution) was added to the buffered glutamate solution to give a final concentration of 0.2 mM. The final glutamate concentration was 4.9 mM. To half of this solution, preincubated at 30 °C, was added glutamate racemase (40 μL, 8.5 units). The two samples were incubated at 30 °C, and the extent of racemization was monitored for 30 min at 204 nm using circular dichroism spectroscopy. The glutamate was derivatized as described below.

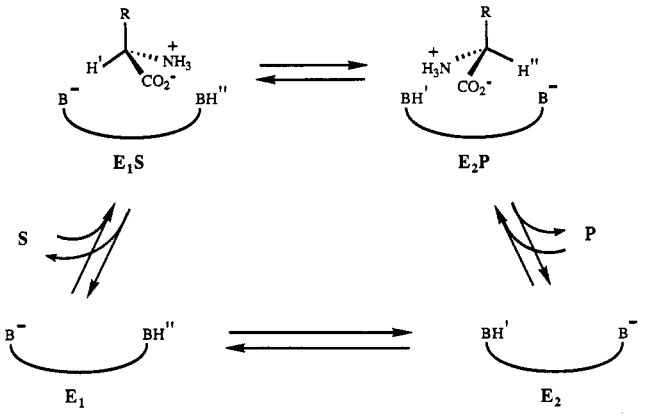

FIGURE 2: General scheme for the two-base mechanism for amino acid racemases. The enzyme E carries two acid-base catalytic groups, B. The substrate S and product P are the amino acid enantiomers.

Reaction of [^{18}O]Glutamate with Dinitrofluorobenzene. Prior to mass spectral analysis, the [^{18}O]glutamate was derivatized with 2,4-dinitrofluorobenzene using a slight modification of the method of Schroeder and LeGette (1953). The [^{18}O]glutamate samples were treated with solid sodium bicarbonate (22 mg). Ethanol (7 mL) was added to each sample, followed by 2,4-dinitrofluorobenzene (DNFB) (31.5 μL of a 100 mg/mL solution). After 1 h at room temperature with intermittent mixing, water (16.5 mL) was added to each sample. The aqueous phase was acidified by the addition of concentrated aqueous HCl, and the 2,4-dinitrophenyl (2,4-DNP) derivative was extracted into diethyl ether (2 × 10 mL). The combined ether extracts were washed with dilute HCl, dried over Na_2SO_4, and evaporated to dryness. The residue from each sample was redissolved in the minimal amount of dimethyl sulfoxide and analyzed by negative ion fast atom bombardment mass spectroscopy. As a control, a sample of unlabeled glutamate was derivatized and analyzed in the same way.

Enzymatic Racemization of (S)-Glutamic Acid in D_2O. (*S*)-Glutamic acid (8 mg, 0.05 mmol) was dissolved in deuterated potassium phosphate buffer (100 mM, 2 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 buffer (1.4 units in 10 μL). The tubes were incubated at 37 °C, and the progress of the reaction was monitored using ^1H NMR spectroscopy (500 MHz).

"One-Base" versus "Two-Base" Mechanism Determination: (a) Racemase Reaction. Buffered solutions of (*R*)- or (*S*)-glutamic acid (17 mM) in D_2O containing 50 mM sodium phosphate were prepared as follows. A solution containing (*R*)- or (*S*)-glutamic acid (50 mg) and dibasic

[2] Abbreviations: MTPA, α-methoxy-α-(trifluoromethyl)phenylacetic acid; MTPA-Cl, α-methoxy-α-(trifluoromethyl)phenylacetyl chloride; ATP, adenosine triphosphate; NMR, nuclear magnetic resonance; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; CD, circular dichroism; TLC, thin-layer chromatography.

sodium phosphate (142 mg) in D_2O (20 mL) was freeze-dried after the pD was adjusted to 8.0. The residue was dissolved in D_2O (20 mL), freeze-dried, and redissolved in 20 mL of D_2O containing dithiothreitol (2 mM).

The solutions were kept under argon at 30 °C. Two control samples (3 mL each) were removed by syringe from each solution and immediately acidified by the addition of concentrated perchloric acid (75 μL). To one of the acidified control samples (from each original solution) was added racemase (1.3 μL; 0.5 unit in a solution in 50 mM triethanolamine hydrochloride buffer, pH 7.0). The racemization reactions were initiated by the addition of glutamate racemase (6 μL; 2.5 units in 50 mM triethanolamine hydrochloride buffer, pH 7.0) to the nonacidified solutions (14 mL each). Portions of these solutions (3 mL each) were removed at timed intervals and then quenched with concentrated perchloric acid (75 μL).

(*b*) *Isolation of Glutamate.* Each of the acidified samples was passed through an ultrafiltration membrane [10 000-MW cutoff, from Centricon-10 concentrators (Amicon, Beverly MA)]. The filtrate was neutralized by the addition of KOH (2 M) and kept at 4 °C overnight. The precipitated $KClO_4$ was then removed by centrifugation. The supernatant was diluted to 15 mL with H_2O and the solution was applied to a column (10 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 (0.5 N, 40 mL). The fractions containing glutamic acid (as indicated by ninhydrin staining on TLC plates) were divided into two equal portions, and each was evaporated to dryness.

(*c*) *Derivatization of Glutamate.* Freshly-distilled acetyl chloride (3 mL) was added to anhydrous methanol (30 mL) at −78 °C under argon, and the mixture was allowed to warm to room temperature. Samples of solid glutamic acid (prepared as above) were dissolved in the acidic methanol (1.5 mL) and transferred quickly to 2-mL screwcap vials fitted with Teflon–silicone septa. Each sample was heated at 73 °C for 3 h and then allowed to cool to room temperature. The vial was vented with a needle, and the solvent was removed by placing the samples under high vacuum. Each sample of glutamic acid (approximately 26 μmol) was dissolved in $CHCl_3$ (1 mL, dried by passage through a column of neutral alumina). Triethylamine (11 μL, 80 μmol, freshly distilled from calcium hydride) was added, followed by (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (10 μL, 52 μmol, distilled). The solution was left at room temperature for 1 h with intermittent mixing. The sample was shaken with H_2O (0.5 mL), dried over Na_2SO_4, and applied to a column of silica gel (2 mL). After the column was washed with $CHCl_3$ (4 mL), the derivative was eluted with ethyl acetate (20% v/v in $CHCl_3$, 5 mL). The diastereomeric composition of each sample was assessed by capillary gas chromatography using a DB-1701 capillary column (30 m).

(*d*) *Gas Phase Chromatography/Mass Spectral Analysis.* The deuterium content of each diastereomer was determined using capillary gas phase chromatography/mass spectral analysis (GC/MS) using the same column described above. Mass spectra were recorded using a JEOL AX-505H mass spectrometer interfaced with a Hewlett Packard 5890 gas chromatograph. Samples were subjected to chemical ionization using isobutane as the reagent gas. The mass spectrometer was operated in the single ion monitoring mode to detect the protonated derivatized molecule (MH^+) and its first isotope peak, i.e., m/z 392 and 393, respectively. The mass spectral analyses were performed by Dr. Andrew Tyler (Harvard University Mass Spectrometry Facility, Cambridge, MA).

Oversaturation Experiments. Two sets of solutions of varying concentration of (*S*)-glutamic acid (0, 12.5, 25, 50, 100, and 150 mM) in 200 mM potassium phosphate buffer, pH 8, were prepared. One set contained potassium chloride (200 mM) and the other had no added salts. The "high salt" solutions were added to the "low salt" solutions until all samples had the same conductivity (12 mS). To each of the adjusted solutions (2.95 mL) was added dithiothreitol (0.03 mL of a 20 mM solution). After temperature equilibration at 30 °C, glutamate racemase (15 μL; 6.5 units in 50 mM triethanolamine hydrochloride buffer, pH 7.0) was added. The ellipticity at 227 nm was followed for the full time course of the racemization. All data points (including the initial point before the addition of enzyme) 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 after appropriate manipulation (see Results section).

RESULTS

Test for an Acylenzyme Intermediate Using [^{18}O]Glutamic Acid. Glutamic acid enriched with ^{18}O in both of its carboxylate groups was prepared by acid-catalyzed exchange between (*S*)-glutamic acid and [^{18}O]H_2O. After incubation of the isotopically-labeled amino acid with glutamate racemase under multiple-turnover conditions and derivatization of the glutamic acid to increase its volatility, the amino acid derivative was analyzed for loss of ^{18}O label by negative-ion fast atom bombardment (FAB) mass spectrometry.

The [^{18}O]glutamic acid was prepared by heating the amino acid in acidic [^{18}O]H_2O (95 atom % excess). These conditions have been shown to result in very efficient incorporation of labeled oxygen into carboxylate groups of amino acids without racemization (Murphy & Clay, 1979). To minimize the number of required manipulations of the labeled substrate, the acidic solution of [^{18}O]glutamic acid was diluted directly into buffered H_2O without isolation of the amino acid. The amount of [^{18}O]H_2O introduced into the final buffered solution was less than 0.7% by volume and was, therefore, negligible. A portion of the [^{18}O]glutamic acid solution was incubated with glutamate racemase, while a control sample was incubated without enzyme. The racemization of the labeled (*S*)-glutamate was followed at 204 nm by CD spectroscopy. After enough time had elapsed such that each molecule of glutamic acid had interacted with the enzyme at least 34 times on average (based on the number of units of racemase added), the samples were treated with 2,4-dinitrofluorobenzene under mildly alkaline conditions.

The ^{18}O isotopic enrichment of the dinitrophenyl derivative of glutamate was estimated by negative-ion FAB mass spectrometry. The major peak in the mass spectrum of the unlabeled glutamate derivative appeared at m/z 312, whereas the ^{18}O-labeled starting material sample showed major peaks at m/z 318 and 320, corresponding to the incorporation of three and four heavy oxygen atoms, respectively. The relative intensities of these two peaks indicate a 94% enrichment with ^{18}O in each of the carboxyl oxygens of glutamate. No peaks at m/z 312, 314, or 316 were detected in the mass spectrum of the sample of the derivatized labeled glutamate, indicating the absence of unlabeled, monolabeled, and dilabeled glutamate. The relative ^{18}O contents of the control sample and the racemase-treated sample were determined in a similar manner. The negative-ion FAB mass spectra of the [^{18}O]-

Table I: Ratio of the Signal at m/z 318 to the Signal at m/z 320 for Various Samples of [^{18}O]Glutamate

[^{18}O]glutamate	[^{18}O]glutamate after incubation with racemase[a]	[^{18}O]glutamate after incubation without racemase[a]
0.2887	0.2801	0.2848
	0.2902	0.2812
	0.2881	0.2815
	0.2825	0.2884

[a] (*S*)-[^{18}O]Glutamate (15 μmol, 94% labeled with ^{18}O in the carboxylate oxygens) was incubated in phosphate buffer with or without glutamate racemase. The amino acid was isolated as its dinitrophenyl derivative and subjected to repetitive estimates of ^{18}O content by negative ion FAB MS.

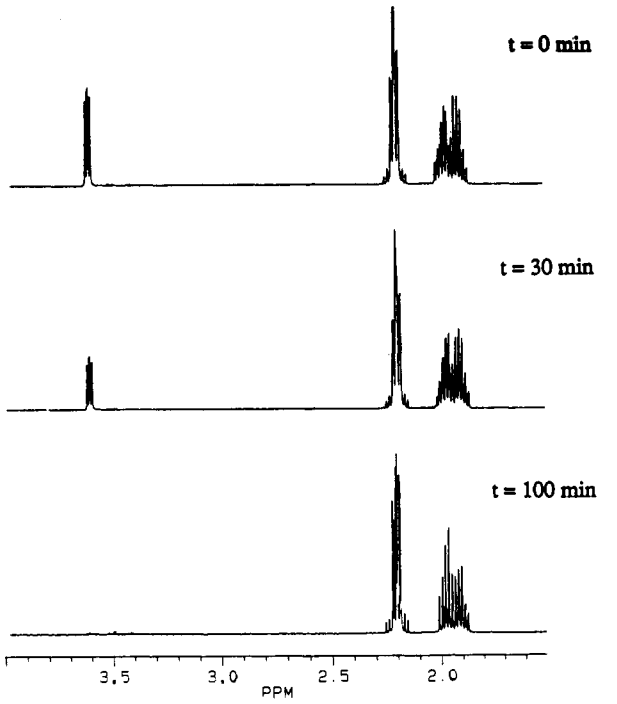

FIGURE 3: ^{1}H NMR spectra of the products from the racemization of (*S*)-glutamate in D_2O. The C-2 proton signals appear at 3.6 ppm.

glutamate control sample and the racemase-treated [^{18}O]-glutamate showed indistinguishable isotopic distributions at m/z 318 and 320, indicating that no loss of ^{18}O label from the [^{18}O]glutamate occurs upon incubation with glutamate racemase. The mass spectral data at m/z 318 and 320 for the control sample and the racemase-treated sample of [^{18}O]-glutamate, as well as that of the untreated [^{18}O]glutamate, are listed in Table I.

Incorporation of Solvent Isotope at the C-2 Position during Enzymatic Racemization. A sample of (*S*)-glutamate was racemized by glutamate racemase in a deuterated buffered solution. The course of the reaction was monitored by ^{1}H NMR, and the spectra are shown in Figure 3. The incorporation of deuterium at the C-2 position is indicated by the disappearance of signals due to the C-2 protons (at 3.6 ppm) and the simplification of the coupling pattern of the signals due to the C-3 protons (at 1.9–2.0 ppm). A control sample containing no enzyme showed no changes in its spectrum under identical conditions.

"One-Base" versus "Two-Base" Mechanism. The extent of enzyme-mediated transfer of the proton of the glutamate substrate to its enantiomeric product was determined for the glutamate racemase reaction in each direction. A catalytic amount of glutamate racemase was added to a buffered solution of the substrate enantiomer in D_2O. Portions of the reaction mixture were removed during the initial phase of the reaction and were quenched by acidification with perchloric acid. The glutamic acid was isolated by ion-exchange chromatography. Following the conversion of the purified glutamates to their dimethyl esters, the enantiomeric mixture was derivatized with the chiral reagent (*R*)-MTPA-Cl (Dale et al., 1969; Powers et al., 1991). Capillary gas phase chromatography/mass spectral (GC/MS) analysis was used to resolve the resulting diastereomers and to determine their deuterium content.

(*a*) *Racemase Reaction.* A buffered solution containing substrate [(*R*)- or (*S*)-glutamic acid] and a thiol-reducing agent was prepared in D_2O. The solution was adjusted to pD = 8.0. [For glass electrodes, pD = $pH_{meter\ reading}$ + 0.4 was assumed (Glasoe & Long, 1960).] The mixture was treated with glutamate racemase and, at timed intervals corresponding to small extents of reaction (from 3% to 10% conversion to product), portions of the reaction mixtures were quenched with perchloric acid.

Control experiments showed that the quench conditions used did not induce any racemization of glutamic acid in the absence of the racemase. However, the acid-induced inactivation of the racemase was found in separate control experiments to be partially reversible upon neutralization of quenched solutions. The racemase was therefore removed from the acidified samples by filtration through an ultrafiltration membrane (10 000-MW cutoff), and the resulting protein-free filtrate was neutralized by the addition of KOH. To be certain that the inactivation of the enzyme was instantaneous, a portion of the reaction solution was first acidified with perchloric acid, and then racemase was added. Upon workup of the reaction mixture, no product was detectable. On the basis of these findings, the procedure for quenching glutamate racemase was deemed to be adequately rapid and irreversible.

(*b*) *Purification of Glutamic Acid.* Potassium hydroxide was used to neutralize the acidified sample because the resulting potassium perchlorate forms a precipitate in aqueous solution. Removal of the precipitated salt greatly reduces the ionic strength of the sample and facilitates the subsequent purification by anion-exchange chromatography. The eluted fractions contained glutamic acid, as indicated by ninhydrin staining, but not phosphate (from the buffer), as indicated by molybdic acid/malachite green staining.

(*c*) *Formation and Resolution of Diastereomers of Derivatized Glutamic Acid.* Heating of the purified glutamic acid in acidic methanol afforded the glutamate dimethyl esters, which were subsequently allowed to react with the chiral resolving agent (*R*)-MTPA-Cl, in the presence of triethylamine. Since the chiral reagent might react with the two enantiomers at different rates, the use of a 2-fold molar excess ensured that the derivatization of both enantiomers of glutamate went to completion. Silica gel flash chromatography was used to isolate the mixture of diastereomers. Baseline separation of the diastereomers was then achieved by capillary gas chromatography, the (*R*,*R*)-diastereomer eluting before the (*S*,*R*)-diastereomer. The relative amounts of the two diastereomers were determined by integration of the gas chromatographic peaks. Multiple injections of a given sample gave results that were reproducible to ±0.5%. In another control experiment, a sample of racemic glutamate was derivatized identically. The two diastereomers in this sample gave equal peak areas when analyzed by GC/MS.

The assessment of the enantiomeric purity of the starting substrates and of the (*R*)-MTPA-Cl is essential for the interpretation of these experiments. For example, since only

Table II: Enzyme-Catalyzed Racemization of (*S*)-Glutamate in D_2O[a]

extent of reaction (% mole fraction of product)	derivatized substrate diastereomer				derivatized product diastereomer		
	area of (MH^+) peak	area of $(MH^+ + 1)$ peak	area of $(MH^+ 1)_{corr}$ peak	% ^1H in substrate (±2%)	area of (MH^+) peak	area of $(MH^+ + 1)$ peak	estimated % ^1H in product
0	100	18.78	−1.17	101.2			
3.6	100	18.95	−1.00	101.0	none detected	100	<4
5.9	100	19.18	−0.77	100.8	none detected	100	<4
7.3	100	19.82	−0.13	100.1	none detected	100	<4
8.6	100	19.61	−0.34	100.3	none detected	100	<4

[a] The substrate (MH^+) peak area was normalized to 100; the % ^1H in the substrate was calculated as $(MH^+)/[(MH^+) + (MH^+ + 1)_{corr}]$, where $(MH^+ + 1)_{corr} = (MH^+ + 1) - (0.1995)(MH^+)$. The product $(MH^+ + 1)$ peak area was normalized to 100.

Table III: Enzyme-Catalyzed Racemization of (*R*)-Glutamate in D_2O[a]

extent of reaction (% mole fraction of product)	derivatized substrate diastereomer				derivatized product diastereomer		
	area of (MH^+) peak	area of $(MH^+ + 1)$ peak	area of $(MH^+ + 1)_{corr}$ peak	% ^1H in substrate (±2%)	area of (MH^+) peak	area of $(MH^+ + 1)$ peak	estimated % ^1H in product
0	100	20.63	0.68	99.3			
4.6	100	19.95	0	100.0	none detected	100	<4
7.0	100	20.33	0.38	99.6	none detected	100	<4
8.8	100	21.03	1.08	98.9	none detected	100	<4
10.8	100	20.86	0.91	99.1	none detected	100	<4

[a] The substrate (MH^+) peak area was normalized to 100; the % ^1H in the substrate was calculated as $(MH^+)/[(MH^+) + (MH^+ + 1)_{corr}]$, where $(MH^+ + 1)_{corr} = (MH^+ + 1) - (0.1995)(MH^+)$. The product $(MH^+ + 1)$ peak area was normalized to 100.

a small percentage of product is allowed to form in the racemase reaction, even a slight contamination of the starting substrate with the product enantiomer could account for a significant fraction of the total product in a racemase-treated sample. This, in turn, would result in a biased measurement of the extent of enzyme-catalyzed proton transfer to the product enantiomer. Analogously, an enantiomeric impurity in the (*R*)-MTPA-Cl would produce small amounts of the (*R*,*S*)- and (*S*,*S*)-diastereomeric cross-over products, again resulting in an overestimation of the extent of proton transfer. Finally, it is crucial that no racemization occurs at either chiral center during the coupling reaction or during the subsequent handling of the glutamate diastereomers. To investigate these possibilities, each glutamate enantiomer was derivatized with (*R*)-MTPA-Cl and subjected separately to capillary GC. In either case, only one product peak was detected (even with large sample loadings on the GC column), and the diastereomeric contamination was estimated at less than 0.3%. Thus, the (*R*)- and (*S*)-glutamate and the (*R*)-MTPA-Cl were considered enantiomerically pure, and epimerization of the diastereomeric adducts that were formed from these reagents was deemed to be negligible.

(*d*) *Determination of Deuterium Content in the Glutamate Diastereomers.* The glutamate diastereomers were separated, and the isotopic content of these diastereomer was determined, using an interfaced GC/MS with selected ion monitoring at m/z values of 392 (MH^+; 2-protiated diasteromers) and 393 ($MH^+ + 1$; 2-deuterated, or ^{13}C-containing, diastereomers). Chemical ionization was employed because this induces very little fragmentation of the diastereomeric glutamate derivatives. The fraction of the ion current in the $MH^+ + 1$ peak (at m/z 393) of the substrate diastereomer that is due to the natural abundance of heavier isotopes (19.951% of ion current in the corresponding MH^+ peak (at m/z 392)) was subtracted from the total ion current of the substrate diastereomer at m/z 393. This corrected value, $(MH^+ + 1)_{corr}$, reflects the quantity of that diastereomer that contains solvent-derived deuterium. The percentage of 2-protium[3] in a given diastereomer can then be calculated as $(MH^+)/[(MH^+) + (MH^+ + 1)_{corr}]$. To assess the reproducibility of mass spectroscopic determinations of the isotopic content of derivatized glutamate, a control sample of unlabeled (*R*,*S*)-glutamate was derivatized and subjected to multiple measurements. The results indicated that the calculated isotopic contents are reproducible to within approximately ±2%.

The samples from the enzyme-catalyzed racemization of glutamate in D_2O showed efficient incorporation of deuterium into the product and no detectable incorporation of deuterium into the remaining substrate, regardless of which enantiomer was used as the substrate. A summary of the GC/MS data and the calculated isotopic content of the derivatized glutamate as a function of the extent of the glutamate racemase reaction in D_2O with (*S*)-glutamate as substrate is shown in Table II. A summary of the GC/MS data and the analogous analysis for the complementary experiment in which (*R*)-glutamate served as the substrate is presented in Table III. From these data, however, it was not possible to calculate the percentage of product diastereomer containing protium at the 2-position, because the MH^+ peak for the product diastereomer was undetectable (we estimate that peaks corresponding to levels of 4% 2-protium-containing diastereomer could be detected, if present).

Oversaturation Experiments. A series of solutions of varying concentrations of glutamic acid (12.5–150 mM) in 200 mM potassium phosphate buffer, pH 8, were prepared and adjusted to constant ionic strength with KCl. These solutions were then treated with glutamate racemase, and the full course of the racemization reaction was monitored by CD spectroscopy at 227 nm.

The oversaturation phenomenon is most readily visualized by plotting the time course of racemization at all substrate concentrations on a single graph. This requires the scaling of both the ellipticity axis and the time axis, to account for the different substrate concentrations. Thus, the ratio of the measured ellipticity to the initial ellipticity (ellipticity/ellipticity$_{t=0}$, which is essentially the fraction of material racemized) is plotted against the ratio of the reaction time to the initial substrate concentration (time/$[S]_0$). For an enzyme that shows normal saturation behavior, a plot of this type will produce a series of curves that lie on top of one another (at substrate concentrations at which the enzyme is fully satu-

[3] Calculated values greater than 100% will occur when the $MH^+ + 1$ peak is smaller than theoretically possible (assuming a natural abundance of isotopes) and are attributed to small errors in the mass spectral measurements.

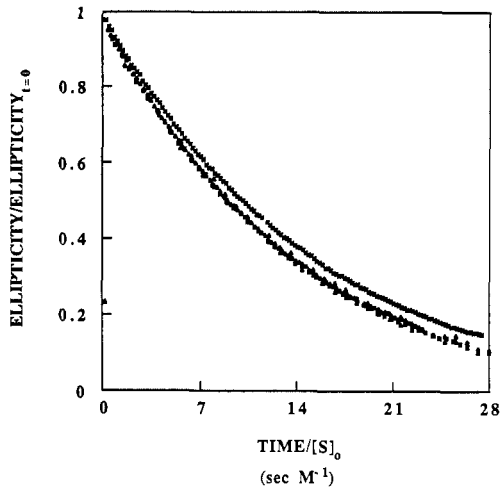

FIGURE 4: Reaction progress curves for the racemization of (*S*)-glutamate by glutamate racemase. The lower line shows the superposition of the normalized progress curves for (*S*)-glutamate at 12.5, 25, 50, and 100 mM. The upper line shows the normalized progress curve for (*S*)-glutamate at 150 mM.

rated). For an enzyme that displays the oversaturation phenomenon (such as proline racemase), a series of curves will be produced with slopes that become shallower with increasing substrate concentrations [see Figure 4 of Fisher et al. (1986)].

The results obtained with glutamate racemase are shown in Figure 4. The progress curves for the racemization of (*S*)-glutamate at 12.5, 25, 50, and 100 mM concentrations are completely superimposable. Only at substrate concentrations as high as 150 mM does the curve begin to deviate significantly. This deviation may indicate the onset of the oversaturation regime, where the interconversion of the two free enzyme forms becomes kinetically significant. An observed nonlinearity of the molar ellipticity values of glutamate precluded measurements at concentrations higher than 150 mM. Similar measurements at lower glutamate concentrations (3 mM to 12.5 mM, concentrations at which the enzyme is still fully saturated) also give overlapping progress curves (data not shown).

DISCUSSION

The previous paper in this series (Gallo & Knowles, 1993) describes experiments that indicate that glutamate racemase does not require any cofactors for the labilization of the relatively nonacidic 2-proton of glutamic acid. One way that an amino acid racemase might acidify the 2-proton is to replace the electron-rich carboxylate group by forming an acylenzyme intermediate at the active site. The removal of the negative charge may lower the pK_a of the 2-proton by as much as 6 units (Landro et al., 1991). This strategy is employed by the enzyme phenylalanine racemase, which utilizes ATP in activating the carboxyl group of the substrate for transfer to an active site cysteine, generating a thioester intermediate (Kanda et al., 1989). Following racemization, the ester bond is hydrolyzed, and the product is released. Although glutamate racemase has no ATP requirement, it is conceivable that an acylenzyme intermediate is also formed against a presumably unfavorable equilibrium constant. Reactions of this type are catalyzed by glycine reductase (Arkowitz & Abeles, 1989) in which ^{18}O atoms in the carboxylate of glycine are lost during conversion to product and by acetylcholinesterase (Bentley & Rittenburg, 1954), which catalyzes the exchange of ^{18}O from solvent into the carboxyl group of acetate. Since the exchange of the carboxylate oxygens of glutamic acid with H_2O is extremely slow (Samuel & Silver, 1965), especially at neutral pH, enzyme-catalyzed washout of ^{18}O from the 1-carboxylate of glutamate would provide support for the existence of an acylenzyme intermediate in the glutamate racemase reaction. However, no racemase-catalyzed washout of oxygen label was observed in experiments using (*S*)-[^{18}O]glutamate as the substrate. These results suggest that there is no glutamyl-enzyme ester or thioester intermediate formed in the reaction catalyzed by glutamate racemase. The existence of a covalent intermediate cannot absolutely be ruled out, however, since it is possible that the labeled H_2O molecule that is generated upon formation of an ester or thioester intermediate does not exchange with bulk solvent and that hydrolysis of the intermediate simply reintroduces the isotopic label.

The observation that the racemization of glutamate results in solvent isotope incorporation at C-2 strongly supports the notion that the reaction proceeds by a deprotonation/protonation mechanism. This observation, however, does not provide any information as to the nature or number of enzymic bases responsible for this reaction.

Two mechanisms have been suggested for enzyme-catalyzed racemization or epimerization reactions that proceed by proton abstraction (Rose, 1966; Cardinale & Abeles, 1968). These have been termed the "one-base" mechanism and the "two-base" mechanism (Figure 1), and they can be distinguished on the basis of the patterns of solvent deuterium incorporation into the substrate and the product. Enzyme-catalyzed transfer of substrate protium to the product in the presence of solvent D_2O is explained most simply by a one-base mechanism. In a two-base mechanism, however, the 2-hydrogen of the product must be derived solely from the solvent. When (*S*)-glutamate is incubated with glutamate racemase in D_2O, essentially all of the product that was formed under initial velocity conditions is labeled with deuterium: no proton transfer from the substrate to the product can be detected. Moreover, no deuterium is detected in the reisolated (*S*)-glutamate substrate. This result is consistent with a two-base mechanism for the glutamate racemase reaction. However, this finding could also be explained by a one-base mechanism in which a single catalytic base abstracts the 2-proton from the substrate (*S*)-glutamate and then (in its acid form) undergoes rapid proton exchange with solvent during the lifetime of the transiently-formed intermediate. To observe no deuterium incorporation into remaining substrate, the intermediate would have to collapse preferentially to the product, (*R*)-glutamate. If glutamate racemase does indeed operate via a one-base mechanism, then, when the enantiomer, (*R*)-glutamate, is used as the substrate in the glutamate racemase reaction, the reisolated (*R*)-glutamate should contain significant levels of deuterium. To distinguish between this latter mechanism and the two-base mechanism, the glutamate racemase reaction was carried out in the opposite direction, i.e., with (*R*)-glutamate as the substrate. The reisolated starting material again contained no detectable deuterium, whereas the 2-hydrogen of the (*S*)-glutamate product was fully labeled with deuterium. On the basis of these findings, a one-base mechanism for glutamate racemase can be excluded. The experimental results are consistent only with a mechanism involving two catalytic bases, neither of which can exchange its proton with bulk solvent while glutamate is bound at the active site.

Since no deuterium was detected in the reisolated starting material regardless of which enantiomer served as the substrate, each of the two catalytic bases is likely to be monoprotic. If a polyprotic catalytic base such as lysine were present, it should catalyze the incorporation of solvent isotopic

label into the substrate enantiomer, on the basis of free rotation about the lysine carbon–nitrogen bond and barring the existence of a large isotope effect upon reprotonation of the enzyme-bound intermediate. For instance, the mechanism of mandelate racemase was recently shown to involve two catalytic bases (Powers et al., 1991) one of which is a lysine residue (Landro et al., 1991; Neidhart et al., 1991). When the 2-proton of (*S*)-mandelate is abstracted by the lysine base, approximately two out of three molecules that collapse back to substrate from the putative carbanion intermediate do so with the incorporation of deuterium. Since the relative frequency of deuterium incorporation reflects the statistical distribution of deuterium on the lysine amino group, the reprotonation of the intermediate by the catalytic lysine in the reaction of mandelate racemase is evidently not subject to a kinetic isotope effect. In proline racemase, the two bases are believed to be monoprotic cysteine residues, a view that is supported by the absence of any solvent isotope incorporation into recovered starting material (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975). Analogously, the data presented in this paper lend support to the notion that glutamate racemase also employs two cysteine residues as the catalytic bases (Gallo & Knowles, 1993).

The two-base mechanism proposed for glutamate racemase demands that there be two enzyme forms that differ by the protonation states of the active site bases (E_1 and E_2 in Figure 2). It is likely that each of these bases is the specific proton acceptor for only one of the glutamate enantiomers and that the enzyme must be in the proper protonation state to convert a given substrate enantiomer to product. The interconversion of the two enzyme forms is therefore necessary for turnover to occur. The interconversion could spontaneously take place following product release (similar to E_2 going to E_1 via the lower pathway in Figure 2), or it could be brought about by reconverting a product molecule back to substrate (a non-productive process, following the upper pathway in Figure 2). The oversaturation experiments provide one method for examining whether the interconversion of the two free enzyme forms is kinetically significant. In the case of proline racemase, at high concentrations of proline (16–190 mM, well past that required for enzyme saturation), the net rate of reaction under reversible conditions actually decreases with increasing substrate concentration. This is due to a significant amount of "recapturing" of the product-handling form of the free enzyme (E_2) before it isomerizes to the substrate-handling form (E_1). Given the mechanistic similarities between these two enzymes, it seemed possible that glutamate racemase would show similar behavior. However, no effects on the net rate of reaction due to increasing substrate concentration (up to 100 mM) were observed. This finding is somewhat surprising in light of the fact that the K_m value for glutamate racemase is one-tenth of that for proline racemase, and one might therefore suspect that oversaturation effects might be observed at lower substrate concentrations with glutamate racemase. The present results suggest, however, that the rate of interconversion of the two free enzymes forms in glutamate racemase occurs significantly faster, compared to the racemization reaction rate, than with proline racemase. The observation of a small decrease in the net rate of glutamate racemization at 150 mM substrate levels may indicate the onset of the oversaturation regime. This concentration is, however, well below a "peak-switch concentration" (that is, the substrate concentration at which the rate-limiting step becomes the interconversion of the two forms of the free enzyme; Fisher et al., 1986). The difference in the behavior of these two enzymes may be due to the fact that proline racemase is a dimer, whereas glutamate racemase is a monomer. It is also conceivable that the buffer used (phosphate) can catalyze the interconversion of the two forms of glutamate racemase.

We have found evidence against acyl-intermediate formation in the glutamate racemase reaction. Furthermore, the incorporation of solvent deuterium at the C-2 position of enzymatically racemized glutamate argues for a deprotonation/protonation mechanism. Deuterium incorporation experiments further indicate that a "two-base" mechanism is operative, and that the bases are likely monoprotic. Finally, the two forms of the enzyme, differing in protonation state, were found to be kinetically insignificant.

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