Studies on the Catalytic Acid/Base Residues of Glutamate Racemase

by

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Department of Chemistry

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THE UNIVERSITY OF BRITISH COLUMBIA

July, 1999

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Date Oct. 14 1999
Abstract

Glutamate racemase from *Lactobacillus fermenti* (murL, E.C. 5.1.1.3) is a cofactor-independent enzyme which catalyzes the interconversion of the enantiomers of glutamate using Cys73 and Cys184 as the general acid/base catalysts. A cysteine thiolate abstracts the \( \alpha \)-proton from one face of glutamate and the other cysteine thiol delivers a proton to the opposite face. The roles of the cysteine residues are explored using the two glutamate racemase mutant enzymes, C73S and C184S. The mutants retain the ability to racemize glutamate with specificity constants \( \sim 10^3 \)-fold lower than those of wild type enzyme. The mutant-catalyzed dehydration reaction of \( N \)-hydroxyglutamate, a one-base requiring reaction, is used to determine which cysteine residue acts on each enantiomer of glutamate. With \( D \)-\( N \)-hydroxyglutamate, the C73S mutant is a poorer catalyst than the wild type enzyme, whereas the C184S mutant is a better catalyst. The opposite trend is observed with \( L \)-\( N \)-hydroxyglutamate. The results suggest Cys73 is the residue responsible for the deprotonation of \( D \)-glutamate and Cys184 is responsible for the deprotonation of \( L \)-glutamate. Furthermore, the \( V_{\text{max}}/K_m \) isotope effect for the C73S mutant in the \( D \rightarrow L \) reaction direction is larger than that observed for wild type enzyme and smaller in the \( L \rightarrow D \) reaction direction. The opposite trend is observed for the C184S mutant. Presumably, an asymmetry in the reaction profile is induced by the mutation making the deprotonation step involving the serine residue more cleanly rate determining. This result supports the assigned roles for each of the cysteine residues of glutamate racemase.

Further experiments explore the importance of the four strictly conserved residues, Asp10, Asp36, Glu152 and His186, by preparing appropriate mutant enzymes. The effect of each of the D36N and E152Q mutations is to reduce \( k_{\text{cat}} \) by a modest factor of \( \sim 2 \)- to 3-fold and suggests these residues are not important to catalysis. They do appear to be involved in binding since there is an increase in the \( K_m \) value for each of these mutants. The \( k_{\text{cat}} \) values for the D10N
and H186N mutants, however, are decreased by three orders of magnitude relative to wild type enzyme implying an important catalytic role for these residues. The $V_{\text{max}}$ isotope effects for D10N and H186N are affected in each reaction direction with the ratio between the isotope effects increased to $2.20 \pm 0.18$ for D10N relative to the wild type ratio of $1.40 \pm 0.34$, and decreased to $0.70 \pm 0.05$ for H186N. A possible role for Asp10 and His186 is to stabilize the thiolate form of Cys73 and Cys184, respectively.
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<td>Ac</td>
<td>acetyl group</td>
</tr>
<tr>
<td><em>Amp</em></td>
<td>ampicillin resistance gene</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>aziDAP</td>
<td>aziridino-diaminopimelate</td>
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<tr>
<td>C184S</td>
<td>glutamate racemase with a Cys→Ser mutation at residue 184</td>
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<td>C73S</td>
<td>glutamate racemase with a Cys→Ser mutation at residue 73</td>
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<tr>
<td>CCA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
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<td>Da</td>
<td>dalton</td>
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<td>DAP</td>
<td>diaminopimelate</td>
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<td>DCI</td>
<td>desorption chemical ionization</td>
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<tr>
<td>DH5α</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
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<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ΔE&lt;sub&gt;0&lt;/sub&gt;</td>
<td>difference in zero-point energy</td>
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<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
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<td>E.C.</td>
<td>Enzyme Commission</td>
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<tr>
<td>E152Q</td>
<td>glutamate racemase with a Glu→Gln mutation at residue 152</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
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<td>-----------</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetate (disodium salt)</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylenebis(oxyethylenenitrilo)tetraacetic acid</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>Et$_3$N</td>
<td>triethylamine</td>
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<td>FAD</td>
<td>flavin adenine dinucleotide</td>
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<tr>
<td>AG$^\circ$</td>
<td>thermodynamic energy barrier</td>
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<td>AG$^+$</td>
<td>activation energy barrier</td>
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<td>GC</td>
<td>gas chromatography</td>
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<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
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<td>glutamate racemase with a His$\rightarrow$Asn mutation at residue 186</td>
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<td>HPLC</td>
<td>high pressure/performance liquid chromatography</td>
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<td>INT$_{ox}$</td>
<td>oxidized $p$-iodonitrotetrazolium violet</td>
</tr>
<tr>
<td>INT$_{red}$</td>
<td>reduced $p$-iodonitrotetrazolium violet</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>catalytic rate constant (turnover number)</td>
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<td>$k_{cat}/K_m$</td>
<td>specificity constant, second order rate constant</td>
</tr>
<tr>
<td>$K_i$</td>
<td>dissociation constant for an enzyme-inhibitor complex</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
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<td>$K_m$, app</td>
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<tr>
<td>$k_{obs}$</td>
<td>observed rate constant</td>
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<tr>
<td><em>L. fermenti</em></td>
<td><em>Lactobacillus fermenti</em></td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani medium</td>
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<td>LBHB</td>
<td>low barrier hydrogen bond</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
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<td>$m$</td>
<td>meso</td>
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</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MPP</td>
<td>mono-peroxyphthalic acid</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MurNAc</td>
<td>N-acetylmuramic acid</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
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<tr>
<td>NAD$^+$</td>
<td>nicotinamide adenine dinucleotide, oxidized form</td>
</tr>
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<td>NADH</td>
<td>nicotinamide adenine dinucleotide, reduced form</td>
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<td>NADP$^+$</td>
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<td>NMR</td>
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<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
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<tr>
<td>$P_{wo}$</td>
<td><em>Pyrococcus woei</em></td>
</tr>
<tr>
<td>$\Delta\theta_{\text{max}}$</td>
<td>maximum perturbation in ellipticity</td>
</tr>
<tr>
<td>R</td>
<td>Gas constant, 8.314 J \cdot mol$^{-1}$ \cdot K$^{-1}$</td>
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<tr>
<td>RCPCR</td>
<td>recombinant circle polymerase chain reaction</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>t</td>
<td>triplet</td>
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<tr>
<td>T</td>
<td>absolute temperature in °K</td>
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<tr>
<td><em>Taq</em></td>
<td><em>Thermophilus aquaticus</em></td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>Trien</td>
<td>triethanolamine</td>
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Tris  tris(hydroxymethyl)amino methane  
UDP  uridine diphosphate  
UV  ultraviolet  
$V_{max}$  maximal reaction rate  
Vis  visible  
w/w  weight-to-weight ratio  

Standard Abbreviations for DNA Bases

A  adenine  
C  cytosine  
G  guanine  
T  thymine  

Standard Abbreviations for Amino Acids

A  Ala  alanine  
C  Cys  cysteine  
D  Asp  aspartate  
E  Glu  glutamate  
F  Phe  phenylalanine  
G  Gly  glycine  
H  His  histidine
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Acknowledgements

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Deo Omnis Gloria
Chapter One

Racemases and Epimerases
1.1 Introduction

The chemical environment of the prebiotic universe was exceedingly simple, consisting of small organic molecules such as water, ammonia, methane, hydrogen cyanide and carbon dioxide. Through a process of chemical evolution, which likely involved photolysis by ultraviolet radiation to form reactive free radicals followed by random recombination, progressively more complex organic molecules were generated (Bonner, 1998). Repetition of such a photolysis-recombination sequence of events ultimately produced a wide variety of increasingly more complex and racemic organic molecules. At some point, however, the prebiotic racemic organic medium underwent a spontaneous symmetry-breaking, or “deracemization” process prior to molecular evolution which ultimately generated replicable, information carrying homochiral biomolecules. Thus proteins arose which consist only of L-amino acids, while RNA and DNA accommodate only D-ribose and 2-deoxy-D-ribose monomer units. Utilizing the available homochiral molecules, primitive organisms began to evolve and to compete with one another for survival. As the challenges to survival increased, so did the need for organisms to adapt and diversify, making access to greater stereochemical diversity one of the essential advantages necessary for survival. Biochemical mechanisms thus evolved which selectively reintroduced stereochemical diversity by the racemization of appropriate molecules while maintaining the stereochemical integrity of the majority of the homochiral molecules necessary for the basic survival of the organism.

There is growing evidence of how a diverse stereochemical repertoire may impart an advantage to an organism. Unusual stereoisomers of amino acids, for instance, are found in the outer protective cell wall of bacteria presumably to provide resistance against proteolysis by making the cell wall unrecognizable to foreign enzymes (van Heijenoort, 1996). Two naturally
occurring antibiotics, gramicidin S and tyrocidine (Kurahashi, 1974; Lipman, 1973), both incorporate a D-phenylalanine residue to exploit the inability of some organisms to metabolize the D-configuration. Certain higher organisms, such as the South American frog, *Phyllomedusa* (Daly, et. al, 1992), or the funnel web spider, *Agelenopsis* (Heck, et. al., 1994), secrete neurotoxins consisting of peptides bearing D-amino acid residues. These examples illustrate the importance of stereochemical diversity as an essential advantage for survival in a competitive environment.

1.2 Racemases and Epimerases

![Reaction catalyzed by amino acid racemases and epimerases.](image)

*Figure 1.1 The reaction catalyzed by amino acid racemases and epimerases.*

Most biomolecules are remarkably stable in an aqueous environment, an essential feature for the integrity of the metabolic system of an organism. The racemization of amino acids for instance, is extremely slow under normal conditions with half-lives as high as 3 500 years for aspartic acid and 12 000 years for alanine (Bada, 1984). In order to provide a pool of unusual stereoisomers, a class of enzymes has evolved that catalyze the inversion of stereochemistry in these molecules. These enzymes, classified as members of the isomerase family, include racemases which catalyze the racemization of enantiomers and epimerases which invert the stereochemistry about a single stereocentre in substrates bearing multiple stereocentres (Figure
1.1). Racemases and epimerases (E.C. 5.1) have evolved to act on a wide variety of molecules including amino acids, carbohydrates, and in certain cases, on isolated chiral centres within large peptides such as neurotoxins and antibiotics by a post-translational epimerization process (Tanner and Kenyon, 1998).

In all cases studied thus far, amino acid racemases and epimerases operate via a simple deprotonation/reprotonation mechanism. An analysis, originally outlined by Rose (1966), proposes two possible mechanisms for this process, the one base mechanism and the two base mechanism (Figure 1.2). The one base mechanism (Figure 1.2 (a)) involves a single enzymic base which removes the proton from one face of the substrate to generate a planar carbanionic intermediate. The protonated base then replaces the proton on the opposite face of the intermediate generating the opposite stereoisomer. The alternative two base mechanism (Figure 1.2 (b)) involves two enzymic acid/base residues, one which removes the proton from one face of the substrate and another which reprotonates the opposite face. The two-base mechanism may proceed by either a stepwise mechanism through a carbanion intermediate as shown in Figure 1.2 (b), or by a concerted mechanism in which the two proton transfers are synchronous.

The transfer of an isotopic label from substrate or solvent to the reaction product may be used as an indicator to distinguish between the two reaction mechanisms. A transfer event known as an “internal transfer” is possible with a one base mechanism and occurs when the same proton removed from one face of a molecule is returned to the opposite face. To observe internal transfer requires that the proton does not completely exchange with solvent during the lifetime of the intermediate. The two base mechanism, however, necessarily involves introduction of a solvent derived proton at the α-position of the product with every turnover. Experiments which
Figure 1.2 Schematic representation of a (a) one-base and (b) two-base catalyzed mechanism. The two base mechanism is shown proceeding by a stepwise reaction through a carbanion intermediate. In the two-base mechanism, isomerization between the two enzyme forms, E₁ and E₂ is shown in the bottom portion of the reaction with substrate (S) and product (P) binding to the appropriate enzyme forms. The enzymic base is represented by (B).

monitor the transfer of an isotope may therefore be used to distinguish between a one-base and a two base mechanism.

Amino acid racemases and epimerases may be broadly classified into one of two categories. Enzymes of the first category have an absolute requirement for a cofactor, such as the commonly employed vitamin B6 derived cofactor, pyridoxal phosphate (PLP), while enzymes of the second category are characterized by their cofactor independence. This comprises a
significant difference in the mechanism employed by racemases and epimerases and enzymes of each category will be dealt with separately in the following sections.

1.2.1 PLP-dependent Amino Acid Racemase

Many enzymes which must abstract a relatively non-acidic amino acid α-proton increase the acidity of this proton by forming a Schiff base between the substrate and an electron withdrawing cofactor such as pyridoxal phosphate (PLP) (Figure 1.3) (Soda, et. al., 1986). PLP is initially bound to the enzyme as a Schiff base with an active site lysine residue. Upon substrate binding, a trans-aldimination occurs between the amino moiety of the substrate and the PLP. The deprotonation of the substrate may then readily occur to form a resonance stabilized carbanionic intermediate. Reprotonation on the opposite face of the intermediate followed by trans-aldimination affords the product enantiomer.

![Figure 1.3 Racemization mechanism of PLP-dependent amino acid racemases. An enzymic base is represented by (B).](image-url)
Amino acid racemases exhibiting PLP-dependence are widely distributed throughout nature, and have been found in both prokaryotic and eukaryotic sources. These enzymes include arginine racemase (Yorifuji, et. al., 1971), serine racemase from both Streptomyces (Svensson and Gatenbeck, 1981) and the silkworm Bombyx mori (Uo, et. al., 1998) and amino acid racemases of broad specificity from Pseudomonas (Lim, et. al., 1998). Alanine racemase, found in both Gram positive and Gram negative bacteria (Walsh, 1989), is perhaps the most thoroughly studied enzyme of this class and is the focus of the following section.

1.2.2 Alanine Racemase

The racemization of alanine by alanine racemase (E.C. 5.1.1.1) represents the simplest reaction catalyzed by enzymes that utilize PLP as a cofactor. This enzyme was first identified in Streptococcus faecalis (Wood & Gunsalus, 1951) and has since been found in a variety of both Gram positive and Gram negative bacteria. Bacteria require the D-isomer of alanine in the synthesis of UDP-N-acetylmuramyl pentapeptide (1), a component of the peptidoglycan of the bacterial cell wall which protects the cell from osmotic shock and lysis. The dipeptide D-Ala-D-Ala is produced from L-alanine in two steps by alanine racemase and D-Ala:D-Ala ligase. The D-Ala:D-Ala-adding enzyme subsequently adds the dipeptide to a UDP-sugar-peptide intermediate.
to yield UDP-N-acetylmuramyl-L-Ala-D-Glu-meso-DAP-D-Ala-D-Ala (1), a key intermediate during the initial stage of peptidoglycan synthesis (Adams, 1976). This compound is transported across the inner membrane by conjugation to a lipid derivative and is subsequently added to the growing peptidoglycan layer of the bacterial cell.

Alanine racemase is not restricted to prokaryotes but has been found in other organisms including the fungus *Tolypocladium niveum* (Hoffman et al., 1994) which utilizes D-alanine in the biosynthesis of cyclosporin A. Other eukaryotic organisms which express alanine racemase include various molluscs and crustaceans, such as the freshwater crayfish *Procambarus clarkii*, which require D-alanine to regulate hyperosmotic stress (Fujita, et al., 1997). Enzymes from the latter sources, however, appear to proceed by a unique mechanism since they do not require pyridoxal phosphate.

\[ 	ext{Figure 1.4 A one-base mechanism for alanine racemase. The rectangle denotes the planar PLP intermediate and demonstrates the two different binding sites which each PLP-alanyl enantiomer intermediate may occupy in the active site.} \]
Early experiments on alanine racemase failed to conclusively determine whether the enzyme operated by a one-base or a two-base mechanism. The marked asymmetry in the kinetic constants of alanine racemase from *Escherichia coli* for L- and D-alanine ($K_m(D-aia) = 0.46$ mM, $K_m(L-aia) = 0.97$ mM, $V_{max} (L\rightarrow D)/V_{max} (D\rightarrow L) = 2.3$) was taken as evidence for the existence of separate binding sites in the enzyme, a condition satisfied by the one-base mechanism (Figure 1.4) (Lambert and Neuhaus, 1972). Since an enzyme is an inherently chiral molecule, however, the diastereomeric substrate/product complexes could easily be of different energies accounting for the observed difference in the kinetic values. Furthermore, no transfer of deuterium label to L-alanine was observed upon incubating D-[α-2H]alanine with alanine racemase in H₂O under single turnover conditions (Shen, et al., 1983). The failure to demonstrate internal transfer of the α-hydrogen suggests a two-base mechanism but is also consistent with a one-base mechanism involving rapid exchange of the protonated enzymic base with the solvent.

With the recent structural determination of alanine racemase, evidence is mounting in favour of a two-base mechanism. The X-ray crystal structure of alanine racemase from *Bacillus stearothermophilus* has been determined to 1.9 Å resolution (Shaw, et al., 1997). The enzyme is a dimer of identical 43 kDa subunits each with a PLP cofactor bound via a Schiff base to Lys39. In the presence of substrate, a transaldimination reaction presumably frees Lys39 which is then properly positioned to act as a general base catalyst. Further reports have published the crystal structure of enzyme-inhibitor complexes of alanine racemase (Stamper, et al., 1998; Morollo, et al., 1999). The crystal structure of an adduct of alanine racemase with the inactivator, (R)-1-aminoethyl phosphinic acid (2) bound as a stable aldime with the PLP cofactor, indicates Tyr265, a residue contributed to the active site by the other monomer, is proximal to the α-carbon of the inhibitor and is properly positioned to act as a general base residue. The available
Recent reports have investigated the importance of these two residues by observing the effect of mutating appropriate residues. In one study, the replacement of Lys39 with alanine by site directed mutagenesis effectively abolished racemase activity, however the activity was restored by the addition of methylamine which assumed the role of Lys39 (Watababe et. al., 1999). Studies on the “methylamine-rescued” enzyme helped to establish the importance of Lys39 in the racemization of the enantiomers of alanine. Further experiments examined the interaction between Tyr265 and adjacent arginine and histidine residues (Sun and Toney, 1999). The X-ray crystal structure indicates these residues participate in a hydrogen bonding network which may influence the ability of Tyr265 to act as an effective general base. Studies on mutant enzymes in which the arginine residue was replaced with other amino acid residues supports a direct role for Tyr265 as a general base in the two base mechanism of alanine racemase. These experiments are discussed in further detail in Chapters Four and Five.

1.2.3 Cofactor-Independent Amino Acid Racemases and Epimerases

Despite the low acidity of the \( \alpha \)-proton in unactivated amino acids, many enzymes effect the racemization of amino acids without the aid of a cofactor such as PLP. These enzymes seemingly lack the means to labilize the \( \alpha \)-proton, yet the turnover of substrate proceeds at
reasonably rapid rates. A number of cofactor-independent racemases and epimerases have been
discovered including glutamate racemase, diaminopimelate epimerase, aspartate racemase, 2-
oxothiazolidine-4-carboxylic acid epimerase and surely others which have yet to be identified
(Tanner and Kenyon, 1998). Proline racemase was among the first known cofactor-independent
racemases and much of the early work focused on this enzyme.

1.2.4 Proline Racemase

Proline racemase (E.C. 5.1.1.4), isolated from the anaerobic bacterium Clostridium, catalyzes the interconversion of the enantiomers of proline (Cardinale and Abeles, 1968). The enzyme is believed to be involved in energy production by providing D-proline which is converted to δ-aminovaleric acid by D-proline reductase, a thermodynamically favourable process (Walsh, 1979; Seto and Stadtman, 1976) (Figure 1.5). Although a crystal structure is not available for proline racemase, it is known to consist of a dimer of identical subunits (MW=38 kDa) and contains a single binding site per dimeric unit.

Pioneering work by Cardinale and Abeles (1968) contributed much to our early understanding of this enzyme. It was demonstrated that the reaction proceeds by a deprotonation/reprotonation mechanism, as opposed to a hydride transfer mechanism, based on

![Figure 1.5 The conversion of L-proline to δ-aminovalerate: a key step in the energy production pathway of the bacterium, Clostridium.](image)
Figure 1.6 The proline racemase reaction proceeding by either a (a) one base mechanism or a (b) two base mechanism. The heavy bar represents the five-membered ring of proline.

the observed incorporation of solvent isotope at the C-2 position of proline when the racemization reaction was run in D₂O. A crucial experiment investigated whether the reaction proceeds by a one base or a two base mechanism (Figure 1.6). When the racemization reaction was carried out in D₂O with L-proline as substrate to ~10% completion such that the reaction was essentially irreversible, all of the D-proline contained deuterium at C-2 whereas recovered starting material did not. In terms of a one base mechanism, this result would require that the protonated enzymic base exchanges rapidly with solvent and that the intermediate partitions
towards D-proline more often than towards L-proline. This would predict that if the same experiment were carried out starting with D-proline, deuterium should be incorporated into D-proline more rapidly than into L-proline. However, the experiment with D-proline as the substrate demonstrated that essentially all of the deuterium label was found in the product, L-proline. This result is inconsistent with the one base mechanism. A two base mechanism in which one enzymic base abstracts the proton from one face of proline and the conjugate acid of a second base delivers a solvent-derived proton to the opposite face to provide the product is consistent with the experimental results observed in the two reaction directions.

Cardinale and Abeles also showed that the abstraction of the α-hydrogen is a rate limiting step since a primary kinetic isotope effect was observed in each reaction direction. A primary isotope effect is observed when a C-H bond is broken in a rate determining step of a reaction. Replacement of the hydrogen by a deuterium results in a slower rate due to the lower zero point energy of a C-D bond. The experiment followed the racemization of L-proline by polarimetry. Each enantiomer of proline exhibits an equal but opposite optical rotation at 366 nm allowing facile observation of the extent of the racemization reaction. The optical rotation of a sample of L-proline in H₂O is initially negative (Figure 1.7, solid circles) but as racemization proceeds the rotation approaches zero due to the accumulation of D-proline. The rotation reaches zero once equilibrium has been reached. When the reaction is carried out in D₂O however, the optical rotation, initially negative, becomes transiently positive as the reaction proceeds (Figure 1.7, open circles). The positive optical rotation is a manifestation of an isotope effect. As the reaction proceeds in D₂O, L-proline is converted to deuterated D-proline, the conversion of which back to L-proline is slowed by a deuterium isotope effect. The reaction rate of unlabelled L-proline exceeds that for labelled D-proline resulting in a temporary accumulation of the D-enantiomer and the rotation becomes positive. Complete deuteration of the proline pool results
in the re-establishment of equilibrium and the rotation returns to zero. The opposite trend is expected on racemizing D-proline in D$_2$O if abstraction of the proton is similarly rate limiting in the reverse direction.

Further experiments by Rudnick and Abeles (1975) established the kinetic significance of the two enzyme forms, E$_1$ and E$_2$ (Figure 1.2 (b)) by demonstrating that the proton removed from proline is protected from solvent and does not exchange until product has dissociated. The experiment measured the rate of tritium release from D$_2$L-[α-$^3$H]proline as a function of proline concentration. Tritium release was found to be suppressed as the concentration of D$_2$L-proline increased. This may be rationalized by recognizing that E$_2$ may convert to E$_1$ either directly by proton exchange or indirectly by binding product to give E$_2$P, catalyzing the back reaction and ultimately regenerating E$_1$ (Figure 1.2 (b)). At very high D$_2$L-proline concentrations the direct
interconversion pathway is suppressed, as demonstrated by the decreased release of tritium, which implies the enzyme bound tritium is recaptured by proline. This is only possible in a pathway with two distinct enzyme forms with an energy barrier to interconversion.

In 1986, Albery and Knowles expanded on the work of Abeles and colleagues and carried out a series of isotopic experiments on proline racemase (Fisher, et al., 1986). The two base mechanism suggests there are two forms of the enzyme (E₁ and E₂; Figure 1.2 (b)), one which binds L-proline and the other D-proline. A tracer perturbation experiment was performed to determine the kinetic significance of the two enzyme forms. A large excess of L-proline was added to an enzymatically equilibrated mixture of D,L-[\textsuperscript{14}C]proline. An internal flux of \textsuperscript{14}C label into L-proline was observed immediately following the addition with a slow return to the equilibrium condition. This can be understood by considering that upon addition of unlabelled L-proline, essentially all of the enzyme will be driven to the E₂ form (the D-pro handling form for the purposes of this discussion) which may either isomerize directly to E₁ or bind labelled D-[\textsuperscript{14}C]proline and convert it back to labelled L-[\textsuperscript{14}C]proline and E₁. If the direct interconversion between E₁ and E₂ is kinetically significant, this process will compete with the "indirect" conversion of E₁ and E₂ via proline racemization. Since label is observed to flow into L-proline, the direct interconversion of E₁ and E₂ is kinetically significant and has an estimated rate constant of $10^5 \text{s}^{-1}$.

A second experiment called the oversaturation experiment was also used to establish the kinetic significance of the two enzyme forms (Fisher, et al., 1986). At very high concentrations of proline, the rate of the reaction is observed to decrease as the substrate concentration increases. This occurs because the level of product is high enough such that free E₂ is often recaptured by product before it can isomerize to E₁. The net conversion of substrate to product then becomes limited by the rate of conversion of free E₂ to free E₁.
While the experiments of Cardinale and Abeles established a two-base mechanism for the reaction catalyzed by proline racemase, their results could not determine whether the reaction proceeds by a stepwise mechanism through a carbanion intermediate or a concerted mechanism involving synchronous proton transfers. To make this distinction, a double isotope fractionation experiment was performed in which the reaction was run in each direction in a mixed H₂O-D₂O solvent (Belasco, et. al, 1983). If the reaction proceeds by a stepwise mechanism (Figure 1.8), deprotonation and reprotonation occur in two different transition states. Reprotonation of the carbanion intermediate to product proceeds with delivery of a solvent-derived proton. When the reaction is performed in mixed H₂O-D₂O starting with unlabelled substrate, a solvent isotope effect discriminates against deuterium incorporation into the product because the second transition state is kinetically significant. Protium is incorporated into the product more often than deuterium such that the product has a lower deuterium content than the solvent (Figure 1.8 (a)). If the reaction is repeated in the same solvent but starting with α-deuterated substrate, the abstraction step becomes more cleanly rate-determining and consequently, the reprotonation is less rate-determining (Figure 1.8 (b)). The deuterium content of the product therefore increases because the second transition state, in which the deuterium discrimination is manifested, is less kinetically significant and there is less discrimination against incorporating deuterium into the product. In a concerted mechanism (Figure 1.9), abstraction of a proton or deuteron by one base coincides with delivery of a proton or deuteron, whichever happens to be bound, from the other base. There is no discrimination against deuterium dependent on the nature of the isotope at the other site. The deuterium content of the product will be the same whether substrate is deuterated or not.
Figure 1.8 Stepwise pathway for the reaction catalyzed by proline racemase with (a) normal substrate and (b) deuterated substrate.

When the above experiment was performed starting with either D-[2-¹H]proline or D-[2-²H]proline, deuterium substitution on the substrate did not affect isotopic discrimination in the product, consistent with a concerted mechanism. However, this result is also entirely consistent with a stepwise mechanism in which the enzymic bases are thiol groups of cysteine residues. This is possible because thiols exhibit an inherent discrimination against deuterium of about twofold. The affinity of the enzymic base for deuterium relative to H₂O, known as the fractionation factor, was measured and found to be 0.55 for each enzymic base, consistent with these groups being thiols (fractionation factor = 0.44 - 0.46) as was suggested by Rudnick and
Abeles based on chemical modification work (Rudnick and Abeles, 1975). In fact, most researchers believe proline racemase operates by a stepwise two base mechanism involving cysteine residues which proceeds through a carbanion intermediate. This conclusion is most consistent with all the experimental evidence available for proline racemase.

1.2.5 Glutamate Racemase

\[ \text{L-Glutamate} \rightleftharpoons \text{D-Glutamate} \]

The existence of a glutamate racemizing enzyme was first suspected in 1947 based on the observation that approximately one half of the total glutamate in bacterial cell hydrolysates is D-glutamate (Dunn, et al., 1947). Ayengar and Roberts (1952) subsequently observed that D-glutamate sustained bacterial growth in media where L-glutamate was absent, prompting them to search for and identify glutamate racemase in \textit{Lactobacillus arabinosus}. The enzyme appeared to be PLP-independent based on the ineffectiveness of PLP inhibitors such as hydroxylamine, and the insensitivity of the rate to added PLP. Soda and coworkers confirmed the cofactor-independent nature of purified glutamate racemase from \textit{Pediococcus pentosaceus} by demonstrating the absence of distinctive absorbance patterns in the UV spectrum (Nakajima, et al., 1986). In addition, they showed that sodium borohydride was ineffective at inactivating the enzyme which should occur if there was a dependence on PLP or other commonly employed cofactor such as FAD, NAD\(^+\) or NADP\(^+\). Thiol reducing agents, such as DTT, were required for enzyme activity while thiol blocking reagents inactivated the enzyme unless substrate was
Figure 1.10 The reaction catalyzed by murD, the D-Glu-adding enzyme.

Glutamate racemase (E.C. 5.1.1.3) from the gene murI has since been found in a number of bacterial genera including Bacilli, Staphylococci, Mycobacteria and Escherichia. The enzyme plays a central role in bacterial cell wall construction where it provides D-glutamate for incorporation into the peptidoglycan (Mengin-Lecreux, et. al., 1989). Following racemization of the available pool of L-glutamate by glutamate racemase, D-glutamate is incorporated into a nucleotide peptidoglycan precursor by addition to UDP-N-acetylmuramoyl-L-alanine (UDP-MurNAc-L-Ala), a reaction catalyzed by murD, the D-glutamate-adding enzyme (Figure 1.10) (Mengin-Lecreulx, et. al., 1989; Pratviel-Sosa, et. al., 1991). Glutamate racemase from E. coli is unique in that it is the only known example of an amino acid racemase whose activity is regulated (Doublet, et. al., 1994; Ho, et. al., 1995). The activity of this enzyme has an absolute requirement for UDP-MurNAc-L-Ala, the substrate of the D-glu-adding enzyme. This provides a mechanism to regulate the formation of D-glutamate, avoiding excessive racemization of glutamate unless required so by active peptidoglycan synthesis. An eventual toxicity of D-glutamate on cell metabolism also supports the necessity for such a regulatory mechanism (Caparros, et. al., 1992).
The essential role of glutamate racemase in bacterial cell wall synthesis makes it a potential target for antibiotic design. Recently, however, it has been found that *Staphylococcus haemolyticus* and *Bacillus sphaericus*, two Gram positive species, exhibit two separate pathways for the biosynthesis of D-glutamate (Fotheringham, *et. al.*, 1998). One pathway proceeds through the racemization reaction catalyzed by glutamate racemase and the other involves a D-amino acid transaminase which synthesizes D-glutamate using α-ketoglutarate and D-alanine as substrates. Glutamate racemase may therefore not be a viable target in the search for new antibacterial compounds, at least in Gram positive species. Despite this, glutamate racemase remains an intriguing enzyme from a mechanistic point of view and continues to elicit much interest.

The enzymatic racemization of glutamate may proceed by either a one-base mechanism or a two-base mechanism. (Rose, 1966). Experiments similar to those discussed earlier for proline racemase were performed to distinguish between these two possibilities (Gallo, *et. al.*, 1993b). Incubating L-glutamate with glutamate racemase in D$_2$O under irreversible conditions resulted in the exclusive incorporation of deuterium label in the product. The reverse experiment, in which the substrate was D-glutamate, resulted in the exclusive incorporation of label in L-glutamate. This result is consistent with a two base mechanism in which one enzymic base removes the proton from one face of glutamate and a second residue delivers the proton to the opposite face to generate the product.

There are two readily observed consequences of a mechanism which employs two residues as the general acid/base catalysts. The first is that an overshoot, similar to the one seen for proline racemase, is observed when the reaction is run in either direction in D$_2$O (Tanner, *et. al.*, 1993). A modest $V_{max}$ isotope effect is measured in both reaction directions (2.2 for L-glutamate and 3.1 for D-glutamate) suggesting that C-H bond breaking is at least partially rate limiting. A second inherent feature of the two base mechanism is that there are two enzyme
forms (E₁ and E₂ in Figure 1.2 (b)), each of which abstracts the α-proton of only one of the glutamate enantiomers. The oversaturation experiment was performed to determine if the direct interconversion of the two enzyme forms is kinetically significant as compared to the non-productive capture of product by E₂ to give E₁. Contrary to the result observed with proline racemase, glutamate racemase exhibited no decrease in the net reaction rate at substrate concentrations up to 100 mM (≈300 x $K_m$; $K_m = 0.3$ mM). A small decrease in the net rate at 150 mM substrate indicated the oversaturating condition may have just been reached. The very high concentration of substrate necessary to begin to observe an oversaturation effect suggests that the direct interconversion of the two enzyme forms of glutamate racemase is not kinetically significant.

The reaction catalyzed by glutamate racemase seems to involve a very straightforward deprotonation/reprotonation mechanism. The simplicity of the reaction is obscured by the fact that the rather difficult deprotonation of the non-acidic α-proton proceeds without an apparent means by which the acidity of the proton may be increased. Various mechanisms have been invoked to explain how such a reaction may be accomplished. Precedence based on the reaction catalyzed by phenylalanine racemase led to the suggestion that an acyl enzyme intermediate may be involved in the reaction (Gallo et. al., 1993b). Phenylalanine racemase labilizes the α-proton of phenylalanine by forming a thioester intermediate between the carboxylate group of phenylalanine and an active site cysteine residue, utilizing ATP to activate the carboxylate group of the substrate (Kanda, et. al., 1989). The removal of the negative charge on the carboxylate group increases the acidity of the α-proton and facilitates the deprotonation step. A similar mechanism is conceivable for glutamate racemase although the ATP-independence of this enzyme would require the formation of the acyl enzyme intermediate under unfavourable equilibrium conditions. The validity of this mechanism was examined by testing for racemase
catalyzed washout of $^{18}$O isotope from dicarboxy-$^{18}$O labelled glutamate. Washout was not observed as would be expected if an acyl enzyme intermediate were formed during the reaction mechanism. The existence of a covalent intermediate cannot be ruled out on the basis of this experiment, however, since it is possible that labelled $[^{18}$O]$H_2O$ does not exchange with solvent and is reintroduced on hydrolysis of the acyl enzyme intermediate.

1.3 Stabilization of the Carbanion Intermediate

Mechanistic enzymologists have long recognized that protons $\alpha$ to a carbonyl, carboxylic acid or carboxylate, although labilized to some degree by resonance stabilization are, in the absence of an activation mechanism, insufficiently acidic to explain the rapid reaction rates typical for enzymes such as the cofactor-independent racemases and epimerases (Gerlt, et. al., 1991). Such enzymes proceed with rates on the order of $k_{cat} = 10^4$ to $10^3$ s$^{-1}$ which is equivalent to an activation barrier ($\Delta G^\ddagger$) of $\sim 13$-kcal/mol as calculated by transition state theory.

$$k_{cat} = \frac{k_b T}{h} \cdot e^{-\Delta G^\ddagger/RT}$$

($k_b =$ Boltzmann’s constant; $h =$ Planck’s constant)

An analysis of the $pK_a$ difference of $\sim 15$ between the $\alpha$-proton of an amino acid and the general base involved in the abstraction of the $\alpha$-proton (based on an estimated $pK_a$ of $\sim 25$ (Rios and Richard, 1997) for the amino acid $\alpha$-proton and $\sim 10$ for a typical general base) clearly demonstrates the problem; the thermodynamic barrier is expected to be $\sim 20$ kcal/mol ($\Delta G^\circ = 2.303 \cdot RT \cdot \Delta pK_a$), approximately 7 kcal/mol higher than the observed $\Delta G^\ddagger$ for the enzyme-
catalyzed reaction. This suggests that a mechanism to stabilize the carbanion intermediate is necessary to account for typical $k_{\text{cat}}$ values of $\sim 10^3 \text{s}^{-1}$.

In 1993, Gerlt and Gassman proposed a mechanism to explain the rapid reaction rates typical for enzyme-catalyzed reactions which involve abstraction of $\alpha$-protons of carbon acids (Gerlt and Gassman, 1993a). A generalized enolization reaction (Scheme 1.1) was considered in which a proton adjacent to a carbonyl is removed by a general base catalyst to form an enolate intermediate. They considered the effect of concerted general acid catalysis at the carbonyl oxygen which they argued would increase the acidity of the $\alpha$-proton. This initial proposal was modified when it was pointed out that the argument failed to address the inherent problem concerning the instability of the resulting enol tautomer. The modified proposal added an extra dimension and suggested that a very short, very strong hydrogen bond, commonly known as a low barrier hydrogen bond (LBHB), forms between the oxygen of the enolate tautomer and the general acid catalyst (Gerlt and Gassman, 1993b). The LBHB supplies the necessary energy to stabilize the high energy enolic intermediate.

\[
\begin{align*}
\text{B}^+ & \quad \text{H} \quad \text{C}^=\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\!\![\text{Scheme 1.1}]
\end{align*}
\]
Figure 1.11 Potential energy wells for hydrogen bonds. (a) Double well hydrogen bond, (b) Low barrier hydrogen bond. The horizontal lines show the energy levels for the lowest vibrational energy for hydrogen (upper) and deuterium (lower).

The strength of a hydrogen bond depends on a number of factors including its length, linearity, nature of the environment and the pKₐ’s of the conjugate acids of the atoms which share the hydrogen. A typical hydrogen bond is ~2.8 Å between the two heteroatoms with a heat of formation of ~5 kcal/mol. The hydrogen is essentially covalently bonded to one atom and interacts electrostatically with the other. In addition, an energy barrier exists between the two possible hydrogen positions (Figure 1.11 (a)). As the distance between the atoms decreases, the energy barrier between the two positions drops to the zero point energy level of the proton allowing free movement of the hydrogen atom with the average position in the centre (Figure 1.11 (b)). This situation describes a LBHB with an unusually strong hydrogen bond bearing partial covalent character. The interatomic distance is decreased to ~2.5 Å and the hydrogen bond heat of formation increases to ~15 - 20 kcal/mol. Factors which permit LBHB formation include matched pKₐ’s between the two conjugate acids of the atoms involved in the hydrogen bond interaction, a low dielectric constant of the surrounding medium and a net charge in the hydrogen bonded system (Emsley, 1980; Hibbert and Emsley, 1990).

Although LBHBs have been recognized for some time in certain compounds such as the FHF⁻ anion, their proposed involvement in enzymatic catalysis is a novel development. Gerlt
and Gassman suggested that LBHBs are in fact possible in the low dielectric, sequestered environment of an enzyme active site provided the $pK_a$'s of the general acid catalyst and the OH group of the enol are matched. When substrate initially binds to the enzyme, there is a weak hydrogen bond because of the large difference in $pK_a$'s between the carbonyl oxygen of the substrate and the general acid catalyst. As the reaction proceeds towards the enol, the $pK_a$ of the enol tautomer increases and matches that of the general acid catalyst permitting the formation of a LBHB. Experimental evidence suggests that the $pK_a$'s between the enol and the general acid are in fact matched (Gerlt and Gassman, 1993b). The differential hydrogen bonding of the conjugate base of the general acid catalyst to the O-H group of the enol intermediate relative to the hydrogen bond of the general acid to the carboxyl group of the keto substrate provides the significant reduction in $\Delta G^\circ$.

A number of unique properties allow LBHBs to be readily characterized. These include a highly downfield shifted NMR signal for the proton involved in the LBHB of 17-21 ppm, a perturbation in the IR stretch frequency as well as a low deuterium fractionation factor of 0.3. The unusually low fractionation factor is a result of the decrease in bond order which occurs as a weak hydrogen bond becomes a LBHB, thus discriminating against deuterium incorporation at the more stiffly bonded position. Examples of enzymes exhibiting at least one of the above properties have been used to support the existence of LBHBs in enzyme active sites (Cleland, 1992; Cleland and Kreevoy, 1994; Frey, et. al., 1994; Cleland, et. al., 1998). X-ray crystal structures have also been invoked as evidence for LBHBs in enzymes (Usher et. al., 1994; Landro et. al., 1994). Although the resolution of a crystal structure is not accurate enough to distinguish heteroatom distances between 2.5 Å and 2.8 Å, general acid residues are seen to be properly positioned to be involved in a hydrogen bond.
Opponents to the above argument note that conclusive evidence to support LBHBs in enzymes is lacking (Guthrie, 1996; Ash, et. al., 1997; Warshel, 1998). It has been suggested that the unusual properties of certain hydrogen bonds may be attributed to the stabilization of isolated charges in a less polar environment by the more equal sharing of a hydrogen bonded proton between the two heteroatoms. Such an arrangement may occur in the low dielectric environment of an enzymatic interior. Thus, the observed spectroscopic properties of certain enzymatic hydrogen bonds may be a manifestation of the enzyme environment and not a reflection of the unusual energetic properties of LBHBs (Shan et. al., 1996). Much evidence also rests on X-ray crystal structures of enzyme-inhibitor complexes which exhibit very short hydrogen bond lengths. If LBHBs are in fact formed within these complexes, one would expect very low $K_i$'s for the corresponding inhibitors. The $K_i$'s are modest however, suggesting LBHBs do not form between the inhibitor and the enzyme.

A major assumption of the LBHB theory is that there is a large increase in hydrogen bond energy as the $\Delta pK_a$ between the two heteroatoms sharing the proton approaches zero. Based on simple electrostatic considerations, hydrogen bond strength is expected to increase linearly as $\Delta pK_a$ decreases. However, a positive deviation at $\Delta pK_a=0$ would be expected if the covalent character of a LBHB provides some additional energetic contribution. Herschlag tested this hypothesis by measuring hydrogen bond energies as a function of $\Delta pK_a$ for a homologous series of substituted phthalate monoanions (3) in DMSO and an intermolecular hydrogen bond system between substituted phenols (4) in THF (Shan and Herschlag, 1996; Shan et. al., 1996). The hydrogen bond that forms between the adjacent carboxyl groups of substituted phthalate has been considered to be a LBHB on the basis of a 21 ppm chemical shift in the NMR spectrum, the isotope fractionation factor of 0.56 and the short hydrogen bond observed in X-ray crystal
structures. A positive deviation was not observed at $\Delta pK_a=0$ for either system suggesting there is no additional contribution to the hydrogen bond when the $pK_a$'s are matched. *Ab initio* calculations of hydrogen bond strength in various systems lend further support to the above conclusion that there is no special stabilization associated with matched $pK_a$'s (Scheiner and Kar, 1995). Opponents concede that although hydrogen bonds are an important factor contributing to the stabilization of the intermediate, it is unlikely that a very short, very strong LBHB provides the bulk of the necessary stabilization.

Alternate proposals offer potential mechanisms which may explain the rapid reaction rates without invoking LBHBs. Guthrie and Kluger note that the negatively charged enolate has the potential to be electrostatically stabilized, a contribution which could be particularly large within the low polar environment of an enzyme active site (Scheme 1.2) (Guthrie and Kluger, 1993). Electrostatic stabilization of the enolate dianion with a metal ion or cationic side chain can, based on calculations, provide 12 - 18 kcal/mol of energy, sufficient to account for the
necessary 7 kcal/mol stabilization of the intermediate. There is a trade-off involved however, in that the stabilization of the ion pair must be paid for at the beginning by desolvation of the cationic group. Therefore, binding energy is traded for orientation where binding energy of the substrate could be used to desolvate cationic and catalytic groups. Furthermore, additional stabilization energy may be gained by hydrogen bonds which are strengthened by geometrical changes in going from the ground state to the transition state, preferentially stabilizing the transition state. Finally, multiple hydrogen bond interactions may contribute additively to transition state stabilization rather than relying on a single very strong hydrogen bond (Shan and Herschlag, 1995).

Although this subject continues to generate much discussion within the enzymological community, the issue remains unresolved. More evidence is needed to firmly establish which, if any, of the above proposals is a likely solution to the inherent problem associated with abstraction of non-acidic α-protons by enzymes such as glutamate racemase.

1.4 Aims of this Thesis

Since glutamate racemase is a representative example of the cofactor-independent racemases and epimerases, a thorough investigation of this enzyme contributes to the overall understanding of the broad class of related enzymes. Moreover, the importance of glutamate racemase in cell wall synthesis makes it a potential target for the development of novel antibiotics. Although certain limitations described in Section 1.2.5 hinder the broad applicability of glutamate racemase inhibitors as antibacterial agents, information garnered from inhibition studies may be beneficial in developing inhibitors for related enzymes.
This thesis expands on the earlier studies on glutamate racemase and goes beyond the questions addressed in previous reports by examining the roles of a number of key residues in the active site of glutamate racemase from *Lactobacillus fermenti*. This work has not only established the role of each of the cysteine residues but has also explored further into the enzyme active site identifying amino acid residues which may play an important role in the mechanism of glutamate racemase.

Chapter Two describes studies on rationally designed inhibitors of glutamate racemase. These inhibitors are structurally and mechanistically similar to potent inhibitors of closely related racemases and epimerases. The inhibition properties of two inhibitors are investigated. Attempts to use an irreversible inhibitor to identify the cysteine residues responsible for the deprotonation events are presented, and a novel reversible inhibitor which also acts as an alternate substrate for the enzyme is characterized. The alternate substrate proves to be an invaluable tool in studies described in Chapter Three.

The roles of the two cysteine residues of glutamate racemase are thoroughly examined in Chapters Three and Four. These experiments identify the cysteine residues involved in the deprotonation of each glutamate enantiomer. Studies on mutant enzymes in which one of the cysteines has been replaced by serine establish the function of each residue by affecting the kinetic parameters for an alternate substrate and by perturbing the energetics which govern the reaction.

Finally, Chapter Five examines the effects of individually mutating several strictly conserved amino acid residues of *L. fermenti* glutamate racemase. Some of these residues may assist the deprotonation of glutamate by cysteine by interactions which stabilize the thiolate form. The mechanistic implications of these interactions are discussed.
Chapter Two

Studies on Irreversible and Reversible Inhibitors of Glutamate Racemase
2.1 Introduction

For several years now, the medical and research communities have been tracking an alarming increase in the number of pathogenic bacteria exhibiting resistance to currently available antibiotic treatments. Of particular concern are bacterial strains which display resistance to multiple drug therapies, effectively making infections related to such bacteria untreatable. The rate at which these resistance properties are emerging is attributed to the ability of bacteria to rapidly evolve new antibiotic resistance mechanisms over successive generations and to readily share these strategies with other bacteria (Davies, 1994).

The diversity of resistance mechanisms which have evolved are both remarkable and frightening. These defense strategies include protein “pumps” that actively remove antibiotics which have entered the cell (Nikaido, 1994), enzymes which destroy or inactivate antibiotics within the cell (Davies, 1994), modifications in enzyme targets to reduce their affinity for an antibiotic (Spratt, 1994) and a decreased permeability of the cell membrane to antibacterial agents (Nikaido, 1994). Our available antibiotic arsenal is rapidly being rendered useless as an ever increasing number of bacteria develop drug resistance, prompting an urgent need for the development of new antibiotics which will at least temporarily evade bacterial resistance.

Antibiotics currently tackle three kinds of targets: protein synthesis, DNA replication and cell wall construction. Antibiotics which target cell wall synthesis are particularly attractive since this pathway is unique to bacteria. Mammalian organisms lack the enzymes which are inhibited by such compounds thus minimizing their toxic physiological side effects. The cell wall, or peptidoglycan, is a rigid layer found in both Gram negative and Gram positive bacteria (Figure 2.1) which confers mechanical support to the cell to prevent rupture due to the high
Figure 2.1. Cell wall features of Gram negative and Gram positive bacteria.
internal osmotic pressure. The presence of the cell wall permits pressures as high as 5 atmospheres in Gram negative bacteria and 25 atmospheres in Gram positive bacteria to be tolerated without cell rupture (Kleinsmith and Kish, 1988). The peptidoglycan is composed of a highly cross-linked network of sugar and amino acid residues. Oligosaccharide chains of alternating $\beta$-1,4-linked $N$-acetylglucosamine (GlcNAc) and $N$-acetylmuramic acid (MurNAc) residues are cross-linked by short peptide chains consisting of D- and L-amino acids attached to a MurNAc residue in the carbohydrate backbone of the peptidoglycan (Figure 2.2). A D-alanine residue of one peptide chain is linked to a second amino acid residue of an adjacent peptide chain either directly by a peptide bond, as is the case in Gram negative bacteria and Gram positive Bacilli, or indirectly by a pentaglycine chain, typical of the Gram positive bacterium.
*Staphylococcus aureus*. The nature of the second amino acid varies from species to species but is either *meso*-diaminopimelate (*m*-DAP) or L-lysine. L-Lysine is often found in Gram positive bacteria while *m*-DAP is present in virtually all Gram negative bacteria.

Penicillin, historically one of the most important antibiotics in the treatment of bacterial infections, was discovered serendipitously in 1929 by Alexander Fleming when he noted the antimicrobial activity of a metabolite produced by the mold *Penicillium notatum* (Boyd, 1984). Penicillin inhibits a late stage in the biosynthetic pathway of peptidoglycan leading to bacteria with an incomplete cell wall that readily rupture in hypotonic media. In normal, viable bacteria, glycopeptide transpeptidase catalyzes the cross-linking step of peptidoglycan by forming a peptide bond between an amino acid residue of one peptide chain and the penultimate D-alanine residue of a second pentapeptide chain with loss of the terminal D-alanine residue (Yocum, *et al.*, 1980). Penicillin, however, is a structural analogue of the acyl-D-Ala-D-Ala terminus of the pentapeptide chain of uncross-linked peptidoglycan and specifically inhibits the transpeptidase that catalyzes this final step in cell wall biosynthesis. The highly reactive β-lactam ring of penicillin irreversibly acylates an active site serine residue and renders the enzyme inactive (Figure 2.3).

![Figure 2.3 Mechanism of inhibition of glycopeptide transpeptidase by penicillin.](image-url)
Enzymologists have since recognized that intermediate and transition state analogues are often extremely potent inhibitors of enzymes. Enzymes evolve to stabilize the high energy transition states as these are the most unstable species encountered along a reaction coordinate (Pauling, 1948). This characteristic has been exploited in the search for new more effective and more specific antibacterial agents. Molecules which mimic the structure of the transition state or intermediate often potently inhibit the enzyme and may be engineered to have high specificity towards unique bacterial enzymes to minimize their potentially toxic side effects in mammals. The enzymes involved in peptidoglycan synthesis have remained highly promising targets for the development of low-toxicity drugs with many pharmaceutical companies actively searching for new lead compounds which inhibit proper formation of the bacterial cell wall.

2.2 Amino Acid Racemase and Epimerase Inhibitors

2.2.1 Proline Racemase Inhibitors

The era of racemase and epimerase directed inhibitor design traces back to early work on proline racemase when it was found that various proline analogues with an sp² hybridized centre at the C-2 position, strongly inhibited the enzyme. Presumably, these molecules mimic the planar geometry at C-2 of the transition state structure of proline in the reaction catalyzed by proline racemase. As such, pyrrole-2-carboxylate (5) (Cardinale and Abeles, 1968) and 2-pyrroline-2-carboxylate (6) (Keenan and Alworth, 1974), which bear an sp²-hybridized centre at
the appropriate position, are both potent competitive inhibitors of proline racemase. Further examples of proline racemase inhibitors include aziridine-2-carboxylate (7), an active-site directed inhibitor of proline racemase (Walsh, 1979), which irreversibly alkylates the enzyme following nucleophilic attack by an active site residue to open the strained aziridine ring. These molecules have served as the basis for the development of inhibitors for related cofactor-independent racemases and epimerases.

2.2.2 DAP Epimerase Inhibitors

DAP epimerase, a cofactor-independent enzyme, interconverts the L,L- and meso-diastereomers of DAP which are either incorporated into the peptidoglycan or are shuttled into the L-lysine biosynthetic pathway. Inhibitors to DAP epimerase should thus prove lethal to bacteria while exhibiting little toxicity towards mammalian organisms which lack a biosynthetic L-lysine pathway.

Significant contributions to the development of DAP epimerase-directed inhibitors have been made by Vederas and colleagues. Compounds prepared by this group include N-modified DAP analogues (8 and 9) (Lam, et. al., 1988), phosphonate analogues of DAP (10) (Song, et. al., 1994), heterocyclic DAP derivatives (11) (Abbot, et. al., 1994) and 3-halo DAP (12) (Gelb, et. al., 1990; Baumann, et. al., 1988) as well as aziridino-DAP (13) developed by Gerhart et. al. (1990). Derivatives 9, 10, and 11 exhibit only modest competitive inhibition of DAP epimerase. N-Hydroxydiaminopimelate (8), tested as a mixture of all four possible diastereomers, exhibits potent competitive inhibition of *Escherichia coli* DAP epimerase with a $K_i$ of 5.6 µM, 2 orders of magnitude stronger binding affinity than L,L-DAP ($K_m = 0.26$ mM). Presumably, N-hydroxy DAP is not itself a potent inhibitor of DAP epimerase, rather enzyme catalyzed dehydration
generates an imine in the active site (14) which mimics the sp² hybridization at the C-2 centre of the reaction intermediate. Similarly, 3-halo DAP derivatives are potent competitive inhibitors (IC₅₀ = 4 - 25 µM) since the base catalyzed elimination of HX results in an enamine (15) in the active site which is also an intermediate analogue. Finally, aziridino diaminopimelate (aziDAP) 13 is an example of a potent irreversible inhibitor similar to the proline racemase inhibitor, aziridine-2-carboxylate. AziDAP irreversibly alkylates an active site residue following nucleophilic attack on the reactive aziridine ring. (Figure 2.4).
2.2.3 Glutamate Racemase Inhibitors

There are only two examples of glutamate racemase inhibitors reported in the literature, each of which suffers from either inefficient inhibition of the enzyme or instability of the compound. L-Serine-O-sulfate is reported to irreversibly inhibit *Pediococcus pentosaceus* glutamate racemase by catalyzing the $\alpha,\beta$-elimination of $\text{HSO}_4^-$ to form 2-aminoacrylate in the enzyme active site (Figure 2.5) (Ashiuchi et al., 1993). Although no supporting evidence was presented, the authors suggest nucleophilic attack by a lysine or cysteine residue in a Michael fashion results in enzyme inactivation by alkylation of a crucial active site residue. This mechanism overlooks the fact that 2-aminoacrylate is a potent nucleophile which would suggest that the mechanism of inactivation is different from that proposed by the authors. The compound is a very poor inhibitor of glutamate racemase with a $K_m$ for the elimination reaction of 83.3 mM, compared to the $K_m$ values for D- and L-glutamate of 14 and 10 mM, respectively.

The second reported glutamate racemase inhibitor is aziridino-glutamate (Tanner and Miao, 1994), which inhibits glutamate racemase by the same enzyme-catalyzed ring opening process as presented earlier for aziridine-2-carboxylate and aziDAP (Figure 2.6). Electrospray ionization (ESI) mass spectrometry of glutamate racemase incubated with aziridino-glutamate
Figure 2.6 Glutamate racemase catalyzed ring-opening of aziridino-glutamate.

demonstrates that the mass of the enzyme is increased by an amount equal to the mass of the inhibitor, supporting the proposal that the enzyme is covalently modified by aziridino-glutamate. It was further shown that only one of the two cysteine residues of *Lactobacillus fermenti* glutamate racemase is labelled by the inhibitor. This was done by treating both a denatured enzyme sample labelled with aziridino-glutamate and an unlabelled control with iodoacetate, a thiol-specific reagent. The mass of the inhibitor-treated enzyme increases by an amount corresponding to the combined mass of racemase, inhibitor and one acetate group. The mass of the control sample increases by an amount corresponding to two acetate groups. These results suggest that nucleophilic attack by one of the cysteine residues leads to aziridine ring opening and covalent modification of the attacking cysteine residue. The remaining cysteine residue is free to be labelled by iodoacetate.

The efficiency of aziridino-glutamate as a glutamate racemase inhibitor is significantly compromised by the instability of the compound. At neutral pH, aziridino-glutamate rapidly cyclizes to the γ-lactone (16) with a half-life of ~4 minutes. Although the instability of aziridino-
glutamate prevented a detailed kinetic analysis of the inhibition, the experiments clearly demonstrate that aziridino-glutamate is an active site-directed irreversible inhibitor of glutamate racemase.

In this chapter, further studies on the resolved enantiomers of aziridino-glutamate are described. These experiments were designed to investigate the specificity of the cysteine residues towards each enantiomer. In addition, a new competitive inhibitor of glutamate racemase, which also acts as an alternate substrate for the enzyme, is described.

2.3 Further Studies on Aziridino-Glutamate

In the normal glutamate racemase reaction, bound glutamate is appropriately positioned such that the α-proton of a particular enantiomer is specifically situated proximal to one of the cysteine residues to permit efficient deprotonation. Aziridino-glutamate should similarly be positioned in the active site exposing the aziridine ring of each enantiomer specifically to one of the cysteine residues. It would be interesting to see if a particular cysteine residue is specifically labelled by a given aziridino-glutamate enantiomer. I had hoped to use this information to discern which cysteine residue is proximal to the methylene of the aziridine ring which, in turn, may identify the cysteine residue responsible for the deprotonation of each enantiomer of glutamate.

The experiment involves treating the enzyme with a resolved enantiomer of aziridino-glutamate to covalently modify a specific residue in the active site. The labelled enzyme is then digested into smaller peptide fragments which are analyzed by an appropriate mass spectrometry technique. A covalent adduct on a particular residue will result in an increased mass for that
peptide fragment. The fragment may then be identified by comparison to a control sample of unlabelled enzyme and by a prior knowledge of the expected masses of the peptide fragments. A cysteine-containing peptide which has been labelled by the inhibitor would provide good indication that a particular cysteine residue has been modified by aziridino-glutamate.

To determine the feasibility of this experiment, we initially examined the inhibition of glutamate racemase by the racemic inhibitor. The synthesis of racemic aziridino-glutamate has been described and proceeds in seven steps through 5-hydroxy-2-(fluoromethyl)-2-phthalimidopentane nitrile 21 prepared by the procedure of Gerhart et al. (1990), with the remaining steps involving oxidation of alcohol 21 to yield the protected carboxylate 22, followed by deprotection and aziridine ring closure to afford the final racemic product 24, as described by Tanner and Miao (1994) (Figure 2.7).

The enzyme was inactivated by incubating glutamate racemase with racemic aziridino-glutamate. Due to the instability of the compound at neutral pH, glutamate racemase was added
to racemic aziridino-glutamate immediately following neutralization. An excess of inhibitor (35 mM) was used to ensure that glutamate racemase was completely inactivated following incubation. ESI mass spectrometry of the modified enzyme confirmed that all of the glutamate racemase was labelled with one molecule of inhibitor.

The next step in the approach requires the digestion of the modified enzyme. Aziridino-glutamate labelled enzyme and an unlabelled control were treated with trypsin, a serine protease which cleaves peptides on the carboxyl side of lysine and arginine residues. Glutamate racemase from \textit{L. fermenti} has 6 arginine residues and 14 lysine residues which results in 21 peptide fragments following complete tryptic digestion (Figure 2.8). The exact masses of these fragments along with the masses of incompletely digested fragments were determined using the program, MacProMass (Appendix A). The two cysteine residues, Cys184 and Cys73 of \textit{L. fermenti} glutamate racemase, are the possible sites of modification by aziridino-glutamate. Following complete tryptic digestion, these two cysteine residues are found in peptide fragments corresponding to amino acids 179-196 with a mass of 2005 Da (MH$^+$), and amino acids 68-101 with a mass of 3330 Da.

An invaluable tool for the detection of peptides in the mass range required for this experiment is matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry. In this technique, the peptide mixture is added to a matrix material which strongly absorbs laser light at a wavelength where the peptides are only weakly absorbing (337 nm).
Ionization of peptides into the gas phase is achieved by thermal relaxation of excited matrix molecules which leads to evaporation of the matrix and transfers the non-volatile peptides into the gas phase. This technique provides high ion yields of the intact analyte and permits the ready detection of peptides in the mass range desired for this experiment.

A MALDI mass spectrum of a tryptic digest of unlabelled glutamate racemase is shown in Figure 2.9. Two peptides of mass 2003 ± 2 Da and 3331 ± 3 Da, corresponding to the Cys184- and Cys73-containing peptides respectively, are observed. The Cys73-containing peptide is in a relatively unobstructed area of the spectrum allowing it to be readily detected. The Cys184-containing peptide, however, is in a very obstructed area of the spectrum. Detection of the Cys184-containing peptide is further hampered by the strong dependence of the signal on the quality of the sample preparation making it very difficult to consistently obtain a strong signal.

To confirm that the two peaks at 2005 Da and 3330 Da do in fact correspond to the fragments bearing one of the two cysteine residues, N-ethylmaleimide, a thiol-specific reagent, was used to label a control sample of digested protein. When a cysteine residue is covalently labelled by N-ethylmaleimide, the mass of the peptide fragment increases by 125 Da. Peptides 179-196 and 68-101, which each bear a cysteine residue, are expected to increase in mass by an amount corresponding to the mass of N-ethylmaleimide with final masses of 2130 Da and 3454 Da, respectively. N-Ethylmaleimide treatment indeed results in two new peaks at 2129 ± 2 Da and 3454 ± 3 Da (Figure 2.10) with the 2005 Da and 3330 Da peaks absent. This result confirms the assignment that the 2005 Da and 3330 Da peaks are the Cys184- and Cys73-containing peptides, respectively.

A mass spectrum of glutamate racemase which had been treated with racemic aziridino-glutamate and digested with trypsin is shown in Figure 2.11. Two new peaks at 2165 ± 2 Da and
Figure 2.9 MALDI mass spectrum of a tryptic digest of glutamate racemase. The peaks at 2003 Da and 3331 Da are the Cys-containing fragments, amino acids 179-196 and 68-101, respectively.
Figure 2.10 MALDI mass spectrum of a tryptic digest of glutamate racemase which has been treated with N-ethylmaleimide after digestion. The inset shows an expansion of the region between 1800 Da and 2400 Da. The N-ethylmaleimide modified fragments are the peaks at 2129 Da and 3454 Da.
Figure 2.11 MALDI mass spectrum of a tryptic digest of glutamate racemase which has been treated with aziridino-glutamate prior to digestion. The upper spectrum (a) shows an expansion of the region between 1800 Da and 2400 Da with the unmodified Cys184-containing peptide at 2003 Da and the modified peptide at 2165 Da indicated. The lower spectrum (b) is an expansion of the region between 3100 Da and 3700 Da showing the unmodified Cys73-containing peptide fragment at 3331 Da and the modified peptide at 3488 Da.
3488 ± 3 Da, in addition to the peaks at 2003 ± 2 Da and 3331 ± 3 Da, were detected. The latter peaks result from the modification of the Cys184-containing peptide fragment (2165 Da) and the Cys73-containing peptide fragment (3490 Da) by each of the aziridino-glutamate enantiomers (160 Da). Since each enzyme may only be labelled at one cysteine residue, the remaining Cys-containing peptide fragment remains unmodified. The peak at 2003 ± 2 Da (Cys184-containing peptide fragment) is from an enzyme which has been labelled at Cys73 while the peak at 3331 ± 3 Da (Cys73-containing peptide fragment) is from an enzyme which has been labelled at Cys184. Although all four peaks were detected in the MALDI mass spectrum of racemic aziridino-glutamate treated enzyme, some difficulties were encountered in detecting the Cys184-containing peptide fragment. Numerous attempts were necessary to obtain a sample preparation which permitted detection of this peak. Despite the difficulties encountered with the experiment, we chose to examine the effect of treating glutamate racemase with each of the resolved enantiomers of aziridino-glutamate.

The two enantiomers of aziridino-glutamate were obtained by resolving the two enantiomers of 22 on a chiral HPLC column. The resolved aziridino-glutamate enantiomers were reinjected onto the column to establish that each enantiomer had been resolved to >97% enantiomeric purity. The remainder of the synthesis was identical to that for racemic aziridino-glutamate.

A marked difference in the binding of each of the enantiomers of aziridino-glutamate by glutamate racemase was noted. Aziridino-glutamate prepared from the enantiomer of 22 with the longer retention time on the chiral HPLC column completely inactivates the enzyme at the lowest evaluated concentration of 2 mM while aziridino-glutamate derived from the enantiomer of 22 which eluted first requires up to 20 mM to completely inactivate the enzyme. The degree of enzyme inactivation was established using a coupled enzyme assay by the loss of glutamate
rracemase activity in aliquots taken from the inactivation reaction. Although the actual inhibition constants for each enantiomer could not be measured due to the instability of the compound, the observed difference in binding between the two enantiomers of aziridino-glutamate suggests an asymmetry in the enzyme active site. Furthermore, this difference suggests that the two enantiomers of aziridino-glutamate were sufficiently resolved and that significant racemization did not occur during the subsequent reaction steps.

With each of the resolved enantiomers of aziridino-glutamate available, we examined the alkylation specificity of the enantiomers to determine if each preferentially labels a particular cysteine-containing peptide fragment. A sample of glutamate racemase was incubated with an enantiomer of aziridino-glutamate and subjected to tryptic digestion. The MALDI mass spectrum of the resulting peptide fragments (Figure 2.12) clearly demonstrates that incubation of glutamate racemase with aziridino-glutamate derived from the enantiomer of 22 with the longer retention time results in the loss of the 3330 Da peak and a new peak at 3490 Da, a difference of 160 Da. The peak at 2005 Da remains (2007 ± 2 Da). Only the peptide fragment containing Cys73 appears to have been modified by this enantiomer of aziridino-glutamate. When the same experiment was performed using the opposite aziridino-glutamate enantiomer, the Cys73-containing peptide fragment was detected (3330 Da), however neither a 2005 Da peak nor the 2165 Da peak expected upon alkylation of the Cys184-containing peptide fragment was observed. It is unlikely that any other fragments were alkylated because the remainder of the spectrum was unaffected. Repeated attempts to obtain a sample which permitted detection of this peptide failed.

Due to the difficulties in detecting the Cys184-containing peptide by MALDI mass spectrometry, we chose not to continue with this project. Electrospray ionization mass spectrometry would be an excellent alternative for the analysis of the peptides from a tryptic
Figure 2.12 MALDI mass spectrum of a tryptic digest of glutamate racemase which has been treated with the enantiomer of aziridino-glutamate derived from the enantiomer of 22 with the longer retention time on a Chiralcel OD column. The aziridino-glutamate modified Cys73-containing fragment is at 3490 Da while the unmodified Cys184-containing fragment is at 2007 Da.
digest of glutamate racemase. With appropriate modification of the conditions used to prepare the tryptic digests, namely an increase in the incubation time with trypsin to increase the concentration of the peptide fragments, aziridino-glutamate or a related derivative could still prove useful as an active site probe to determine which enantiomer of the inhibitor is attacked by a particular cysteine residue.

We have succeeded in establishing that racemic aziridino-glutamate can label either cysteine in glutamate racemase and that the resolved enantiomers of aziridino-glutamate do in fact inhibit the enzyme with different binding affinities. It would be useful to determine the inhibition constants, perhaps by performing a stopped flow experiment which would allow the inhibition to be measured before significant decomposition of aziridino-glutamate occurs. Although further experiments could complete the study, we found success with another inhibitor which is described in the following section. This inhibitor is used in experiments described in Chapter Three which help to establish the roles of each of the cysteine residues in the deprotonation of the enantiomers of glutamate.

2.4 N-Hydroxyglutamate: A Competitive Inhibitor of Glutamate Racemase

It has previously been noted that a mixture of the four possible N-hydroxydiaminopimelate diastereomers exhibits potent competitive inhibition of diaminopimelate epimerase \( K_i = 5.6 \mu M \) (Lam, et. al., 1988). It was also reported that N-hydroxy DAP may dehydrate in the enzyme active site to generate the imine which may be the species responsible for the inhibition properties, although evidence for this mechanism was not presented. The imine exhibits planar geometry at the C-2 position, mimicking the sp\(^2\) hybridization at C-2 of the normal reaction intermediate. Since glutamate racemase and
diaminopimelate epimerase are closely related cofactor-independent enzymes, N-hydroxyglutamate may similarly act as a competitive inhibitor of glutamate racemase by formation of an imine in the active site. A covalent adduct between the enzyme thiol and the inhibitor may be in equilibrium with the non-covalently bound imine and may participate in the inhibition mechanism as shown in Figure 2.13 (upper right).

The stereospecific synthesis of N-hydroxy amino acids has been described by Polonski and Chimiak (1979) with the preparation of each enantiomer of N-hydroxyglutamate starting from its respective glutamate enantiomer (Figure 2.14). The reaction proceeds by formation of a Schiff base between the dimethyl ester of glutamate and p-anisaldehyde. The imine is then oxidized to an oxaziridine with monoperoxyphthalic acid (MPP). An advantage of MPP is that the product of the oxidation, phthalic acid, precipitates out from the mixture and is readily removed. Oxaziridine ring cleavage is performed under mildly acidic conditions by treatment with hydroxylamine-hydrochloride. Protonation of the oxaziridine oxygen atom followed by attack at the oxaziridine carbonium ion by free hydroxylamine leads to the formation of dimethyl N-hydroxglutamate and an oxime (Polonski and Chimiak, 1979; Butler and Challis, 1971;
Emmons, 1957). Oxaziridines bearing a C-aryl substituent preferentially undergo C-O bond fission presumably due to the stabilization of the resulting benzylic carbonium ion. The final product, N-hydroxyglutamate, is obtained by acid hydrolysis of the dimethyl ester.

To demonstrate that racemization at the C-2 centre does not occur during the course of the reaction, each enantiomer of N-hydroxyglutamate was hydrogenated over Pd/C to its respective glutamate enantiomer. An enzyme assay was used to determine both the amount of total glutamate and the amount of L-glutamate in the sample. The assay measures the total amount of glutamate in a sample by coupling the formation of L-glutamate by glutamate racemase to the reduction of p-iodonitrotetrazolium violet (INT) which has a maximum absorbance at 500 nm (Figure 2.15). The coupling to INT reduction is necessary to drive the thermodynamically unfavorable formation of α-ketoglutarate by L-glutamate dehydrogenase. By following the reaction to completion, the concentration of glutamate may be determined. A variation of this assay in which glutamate racemase is heat-inactivated before being added to the assay, determines the amount of L-glutamate in each hydrogenation sample. The relative concentration of L-glutamate:total glutamate led to the determination that >94% enantiomeric
Figure 2.15 The continuous spectrophotometric coupled enzyme assay used to measure the kinetics for the formation of L-glutamate by glutamate racemase.

excess is retained and significant racemization of N-hydroxyglutamate does not occur during the synthesis.

Even though the N-hydroxyglutamate samples appear to be >95% pure by $^1$H NMR, small amounts of impurities or starting materials could be present. Most of the impurities were removed by ion exchange chromatography, however glutamate may have a similar retention time as the desired product and may co-elute with N-hydroxyglutamate. An assessment of the contamination by the respective enantiomer of glutamate using the coupled assay with both glutamate racemase and L-glutamate dehydrogenase present found that less than 3% of glutamate was present in each sample as an impurity.

By analogy to N-hydroxydiaminopimelate, N-hydroxyglutamate should competitively inhibit glutamate racemase. Dehydration results in the formation of an imine in the active site which is an analogue of the normal glutamate racemase reaction intermediate. The inhibition constant was determined by following the initial velocity of glutamate racemization in the D → L direction using the coupled enzyme assay described in Figure 2.15. The efficiency of the assay is compromised, however, by the presence of N-hydroxyglutamate which is a known competitive inhibitor of L-glutamate dehydrogenase, one of the coupling enzymes in the assay (Cooper and Griffith, 1979). The inhibition constants for the dehydrogenase in the presence of each
enantiomer of $N$-hydroxyglutamate were measured and found to be $0.19 \pm 0.01$ mM for D-$N$-hydroxyglutamate and $28 \pm 3$ $\mu$M for L-$N$-hydroxyglutamate. The inhibition of the coupling enzyme by D-$N$-hydroxyglutamate is sufficiently weak to permit the measurement of the inhibition constant of glutamate racemase by this enantiomer.

The inhibition of glutamate racemase by D-$N$-hydroxyglutamate was found to be of a competitive nature (Figure 2.16) with a $K_i$ value of $56 \pm 2$ $\mu$M as compared to a $K_m$ value of $0.3$ mM for the normal racemization reaction. This constitutes the first effective competitive inhibitor of glutamate racemase to be reported (Glavas and Tanner, 1997). On extended incubation with saturating amounts of D-$N$-hydroxyglutamate, no irreversible inhibition was detected. Due to the significant inhibition of the coupling enzyme by L-$N$-hydroxyglutamate, the inhibition constant of glutamate racemase by this enantiomer could not be determined. The problem is made even greater by the fact that the $K_i$ value for L-$N$-hydroxyglutamate appears to be smaller than the $K_i$ value for D-$N$-hydroxyglutamate.

![Figure 2.16 D-$N$-Hydroxyglutamate inhibition pattern. D-$N$-hydroxyglutamate concentrations are: 0 $\mu$M (○), 5 $\mu$M (●), 20 $\mu$M (□), 60 $\mu$M (■).]
Since the inhibition of glutamate racemase by \(N\)-hydroxyglutamate is thought to involve the formation of an imine in the enzyme active site, it is likely that the imine would be released into solution where rapid hydration and elimination of ammonia would result in a net conversion to \(\alpha\)-ketoglutarate (Figure 2.13, lower path). To examine the possibility of \(N\)-hydroxyglutamate being an alternate substrate for glutamate racemase, each enantiomer of \(N\)-hydroxyglutamate was incubated with large amounts of glutamate racemase in deuterated potassium phosphate buffer and the reaction was followed by \(^1\)H NMR at 37°C. The intensities of the signals corresponding to the protons of \(N\)-hydroxyglutamate (1.8 ppm, 2.2 ppm, and 3.4 ppm) decreased over time as

Figure 2.17 Time course \(^1\)H NMR spectrum of the enzymatic conversion of \(d-N\)-hydroxyglutamate to \(\alpha\)-ketoglutarate.
signals corresponding to α-ketoglutarate increased (2.4 ppm and 2.9 ppm) (Figure 2.17). The time course NMR spectra clearly demonstrate that either enantiomer of N-hydroxyglutamate is completely converted to α-ketoglutarate. Furthermore, the integral of the H_c signal does not change with respect to those of H_a or H_b as the reaction progresses indicating that racemization does not compete with the elimination reaction.

To determine the kinetic parameters for the elimination reaction of N-hydroxyglutamate catalyzed by glutamate racemase, a coupled enzyme assay was developed. Glutamate racemase catalyzes the conversion of N-hydroxyglutamate to α-ketoglutarate which can be coupled to NADH consumption by L-glutamate dehydrogenase as it catalyzes the formation of L-glutamate from α-ketoglutarate and ammonia. NADH consumption can be conveniently monitored by the decrease in absorbance at 340 nm. This reaction operates in the thermodynamically favourable direction for L-glutamate dehydrogenase. The kinetic parameters for formation of α-ketoglutarate from D-N-hydroxyglutamate were found to be $K_m = 57 \pm 1 \mu M$, $k_{cat} = 0.18 \pm 0.01 \text{ s}^{-1}$, and $k_{cat}/K_m = (3.2 \pm 0.2) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. The similarity between the $K_m$ and the $K_i$ reported above is expected for this type of an inhibition process (Segel, 1975).

Problems were once again encountered when kinetic parameters for L-N-hydroxyglutamate were measured. Significant inhibition of the coupling enzyme, L-glutamate dehydrogenase, by L-N-hydroxyglutamate did not allow a full kinetic analysis of the elimination reaction catalyzed by glutamate racemase. However, it was possible to use the coupled assay to determine the second order rate constant by performing the reaction at a single low concentration of L-N-hydroxyglutamate (20 μM). Under these conditions, sufficient L-glutamate dehydrogenase remains uninhibited such that the reaction is coupled. The observed decrease in absorbance at 340 nm as the reaction proceeds to completion was fit to a first order exponential
decay equation, rate = k_{obs}[S], where the rate constant, k_{obs} = \frac{k_{cat}}{K_m} [E], to give a \frac{k_{cat}}{K_m} value of 30 M^{-1} s^{-1} for L-\textit{N}-hydroxyglutamate (the error in enzyme concentration contributes to an error of approximately 10% in the \frac{k_{cat}}{K_m} values). The \frac{k_{cat}}{K_m} value was similarly measured for D-\textit{N}-hydroxyglutamate to give a value of 3.7 \times 10^3 M^{-1} s^{-1}. This agrees with the second order rate constant for D-\textit{N}-hydroxyglutamate consumption determined above. The difference in the kinetic parameters between the two enantiomers of \textit{N}-hydroxyglutamate mirrors the difference observed in the binding between the enantiomers of aziridino-glutamate.

To establish that inhibition of glutamate racemase by \textit{N}-hydroxyglutamate is not due to the presence of a small impurity in the sample, the loss of inhibition may be correlated to the loss of \textit{N}-hydroxyglutamate by enzymatic conversion to \textalpha-ketoglutarate. A sample of D-\textit{N}-hydroxyglutamate (1.2 mM) was incubated with sufficient glutamate racemase such that 75% of the D-\textit{N}-hydroxyglutamate was converted to \textalpha-ketoglutarate leaving 0.3 mM D-\textit{N}-hydroxyglutamate. The reaction was quenched by acidifying the solution and the denatured protein was removed by ultrafiltration. A control reaction lacking enzyme was similarly treated. The sample of D-\textit{N}-hydroxyglutamate was diluted into an enzyme inhibition assay mixture (20 X dilution) such that 0.06 mM of D-\textit{N}-hydroxyglutamate was present in the control and 0.015 mM was present in the experimental sample. The expected rate calculated for the sample with 0.06 mM D-\textit{N}-hydroxyglutamate is 65% the rate of an uninhibited reaction assay while the rate of the sample in which only 0.015 mM D-\textit{N}-hydroxyglutamate remains should be 88% of an uninhibited reaction. The observed rates for the experimental samples were 70% and 91%, respectively. The agreement between the rate of destruction of D-\textit{N}-hydroxyglutamate and the loss of inhibition supports the notion that D-\textit{N}-hydroxyglutamate inhibits glutamate racemase and is not due to an impurity in the sample.
2.5 Conclusion

D-N-Hydroxyglutamate is the first reported effective competitive inhibitor of glutamate racemase. The difference in the second order rate constants between the D and L enantiomers, likely expressed in the binding term, $K_m$, demonstrates that a marked asymmetry exists in the enzyme active site. This difference was also observed in the binding of the enantiomers of aziridino-glutamate although we were unable to establish which enantiomer was the stronger inhibitor. Although glutamate racemase has evolved to bind equally to the enantiomers of glutamate, it is by nature a chiral catalyst and any structural modifications to the respective glutamate enantiomers could manifest themselves quite differently within the enzyme active site, resulting in the observed difference in binding between the enantiomers of aziridino-glutamate and $N$-hydroxyglutamate. This difference in binding presents an opportunity whereby D-based inhibitors with potential antibacterial properties may be developed for enzymes which handle this enantiomer. Presumably, the exclusively L-recognizing enzymes of mammalian organisms would be less severely affected by D-based inhibitors allowing D-based antibiotics with low mammalian toxicity to be developed.
2.5 Experimental Methods

2.5.1 Materials

All buffers and reagents were purchased from Aldrich Chemical Co. or Sigma Chemical Co. unless otherwise stated. Fluoroacetonitrile was prepared by distilling a mixture of fluoroacetamide and phosphorus pentoxide (Pravdic and Keglevic, 1965). Monoperoxyphthalic acid (MPP) was prepared from monoperphthalate magnesium salt hexahydrate by acidifying an aqueous solution with concentrated sulfuric acid to pH < 2 and extracting MPP with diethyl ether. D$_2$O was purchased from Cambridge Isotope Laboratories. Silica gel (230 - 400 mesh, BDH) was used for column chromatography. Diaphorase and L-glutamate dehydrogenase were purchased from Boehringer Mannheim. Trypsin from bovine pancreas was purchased from Sigma. Lactobacillus fermenti glutamate racemase was purified from Escherichia coli DH5$\alpha$ bearing the glutamate racemase encoding plasmid, pKG3 by the procedure described in Chapter Three.

2.5.2 Synthesis of Racemic Aziridino-Glutamate

The synthesis of racemic aziridino glutamate has been described by Tanner and Miao (1994) and proceeds in three steps from 5-hydroxy-2-(fluoromethyl)-2-phthalimidopentanenitrile 21. The preparation of 21 has been described by Gerhart et. al. (1990). The $^1$H NMR and MS data were identical to that previously reported for aziridino glutamate (Tanner and Miao, 1994).
2.5.3 Resolution of the Enantiomers of Aziridino-glutamate

The two enantiomers of 22 were resolved by injecting a sample of 22 dissolved in 4% chloroform/25% ethanol/71% hexanes onto a ChiralCel OD column (250 mm x 4.6 mm, Chiral Technologies Inc.) and eluted using an isocratic gradient of 2% ethanol/hexanes on a Waters 600E HPLC system equipped with a Waters 486 tunable absorbance detector. Resolved enantiomers were reinjected to establish enantiomeric purity. The remaining synthetic steps are identical to those described for the preparation of racemic aziridino-glutamate (Tanner and Miao, 1994).

2.5.4 Modification of Glutamate Racemase by Aziridino-glutamate

Aziridino-glutamate in 1 M potassium hydroxide (15 µL) was diluted into 2 M Trien-HCl pH 8 buffer (35 µL) and the pH was adjusted to pH 7 ± 0.5, as estimated by pH paper, with 2 M hydrochloric acid. Glutamate racemase (0.06 mg, 10 units) was added immediately following neutralization and incubated at room temperature for 1 hour (65 µL total volume). Control samples lacking aziridino-glutamate were similarly treated.

2.5.5 Tryptic Digestion of Glutamate Racemase

Glutamate racemase (65 µL) from the above reaction was diluted into 25 mM Tris-HCl pH 8 buffer containing 1 mM EGTA and 2 mM calcium chloride (500 µL total). Trypsin was added (1:100 w/w ratio of glutamate racemase) and the solution was incubated at 37°C for 5
minutes. Trypsin activity was destroyed by adding 5 mM phenylmethylsulfonyl fluoride and 2 mM EDTA. Stock solutions of 100 mM phenylmethylsulfonyl fluoride in ethanol and 100 mM EDTA were used. The peptide fragments were desalted either by using an Amicon Centricon-3 centrifugal filtration device and exchanging with 0.1% trifluoroacetic acid or by passing the sample through a column of Sephadex G10 resin and eluting with 0.1% trifluoroacetic acid.

2.5.6 N-Ethylmaleimide Modification of a Tryptic Digest of Glutamate Racemase

A tryptic digest of glutamate racemase which had not been labelled with aziridino-glutamate was treated with a solution of N-ethylmaleimide (0.5 mg/mL in ethanol; 6 molar equivalents N-ethylmaleimide:1 molar equivalent glutamate racemase) and incubated for 30 minutes at room temperature. Excess N-ethylmaleimide was quenched with 0.7 mM DTT for 30 minutes. The peptide fragments were desalted using an Amicon Centricon-3 centrifugal filtration device and exchanged with 0.1% trifluoroacetic acid.

2.5.7 MALDI-TOF Mass Spectrometry

Peptide fragments from the tryptic digest (1 μL; 1-10 pmol/μL in 0.1% trifluoroacetic acid) were mixed with a matrix of α-cyano-4-hydroxycinnamic acid (CCA) (9 μL of a saturated solution in 2-propanol/water/formic acid, 2:3:1). Samples were analyzed on a Bruker Biflex MALDI time-of-flight mass spectrometer operated in the reflex mode and externally calibrated with human angiotensin II in CCA matrix. The masses of the peptide fragments were compared to the expected masses determined using the MacProMass program version 2.0β3 (Beckman Research Institute).
2.5.8 Synthesis of *N*-Hydroxyglutamate

*N*-Hydroxyglutamate was prepared by the method of Polonski and Chimiak (1979) with modifications by Kelland *et. al* (1986). These modifications included the use of HONH₂·HCl in the preparation of dimethyl *N*-hydroxyglutamate and the use of silica gel chromatography (25% diethyl ether in dichloromethane) during its purification. The final product of *N*-hydroxyglutamate was purified by elution from a column of AG1-X8 resin (100-200 mesh, formate form) with 0.25 N formic acid. Each enantiomer of *N*-hydroxyglutamate was prepared from its respective enantiomer of glutamate; $^1$H NMR (D₂O, 400 MHz) $\delta$ 3.57 (t, $J = 6$ Hz, 1H), $\delta$ 2.23 (t, $J = 7.5$ Hz, 2H), $\delta$1.87 (m, 2H); MS (MALDI) 165 (MH⁺).

2.5.9 Enzyme Kinetics

2.5.9.1 Assay A

The racemization of D-glutamate by glutamate racemase was monitored using a known continuous coupled spectrophotometric assay (Gallo and Knowles, 1993) that employs L-glutamate dehydrogenase and diaphorase to couple the formation of L-glutamate by glutamate racemase to the reduction of p-iodonitrotetrazolium violet (INT). Rates were followed by monitoring the increase in absorbance at 500 nm using a Cary 3E UV-Vis spectrophotometer. All assays were performed at 30°C in 50 mM Trien-HCl pH 8 buffer with 0.65 mM INT, 5 mM NAD⁺, 25 mM ADP, 37.5 units L-glutamate dehydrogenase, 2 units diaphorase, 0.15 units glutamate racemase and a variable concentration of D-glutamate (1 mL total volume). Racemization was initiated by the addition of D-glutamate. Prior to the addition of substrate, a
background rate was observed, attributed to the direct reduction of INT by DTT present in the glutamate racemase sample. A slight impurity (approximately 2%) of L-glutamate in D-glutamate produced a brief burst in the rate as L-glutamate was converted to α-ketoglutarate by L-glutamate dehydrogenase. Initial velocities were therefore calculated from the slope between 3 and 5 minutes after initiation using a least squares analysis with Cary 3 software version 3.0. The extinction coefficient of reduced p-iodonitrotetrazolium violet at 500 nm was taken at 14 300 M$^{-1}$ cm$^{-1}$.

2.5.9.2 Assay B

The conversion of D-N-hydroxyglutamate to α-ketoglutarate and ammonia was monitored by employing a coupled enzyme assay which uses L-glutamate dehydrogenase to convert α-ketoglutarate and NADH to L-glutamate and NAD$^+$. The assay was performed at 30°C in Trien-HCl buffer, pH 8, with 0.08 mM NADH, 2.5 mM ADP, 2 mM DTT, 5 mM NH$_4$Cl, 12 units L-glutamate dehydrogenase and 3.2 units glutamate racemase to a total volume of 1 mL. The progress of the reaction was monitored by the decrease in absorbance at 340 nm. The slope was calculated by performing a least squares analysis using the Cary 3 software version 3.0. The extinction coefficient of NADH at 340 nm was taken at 6220 M$^{-1}$ cm$^{-1}$.

The $k_{cat}/K_m$ value for L-N-hydroxyglutamate was determined using a similar assay to the one described above at a single low concentration of L-N-hydroxyglutamate (20 μM) and a high concentration of glutamate racemase. The assay was performed at 30°C in 50 mM Trien-HCl buffer, pH 8, with 0.1 mM NADH, 2.5 mM ADP, 2.0 mM dithiothreitol, 10 mM NH$_4$Cl, 12 units L-glutamate dehydrogenase and 280 units glutamate racemase (1 mL total volume). The reaction was followed to completion by monitoring the decrease in absorbance at 340 nm. The resulting
curve was fit to a first order rate equation using the program GraFit, version 3.03 (Erithacus Software Ltd., 1994).

2.5.10 Establishing N-Hydroxyglutamate Purity

To confirm the stereochemical purity of N-hydroxyglutamate, each enantiomer was reduced to the corresponding glutamate enantiomer. Each sample of N-hydroxyglutamate (15 mg) was dissolved in 30% aqueous ammonia (2.5 mL) over 10% Pd/C (2 mg). Hydrogenation was accomplished by reaction under an atmosphere of H₂ (1 atmosphere) for 20 hours at room temperature. The samples were filtered, evaporated to dryness, redissolved in 0.1 M potassium phosphate buffer, pH 8 (0.5 mL) and applied to a column of Dowex AG1X8 resin (formate form, 100-200 mesh). Glutamate was eluted with 0.25 M formic acid, lyophilized to dryness and redissolved in water (~30 mM final concentration). Assay A, run to completion, was used to determine the total amount of glutamate present in the sample (0.015 mM glutamate and 12 units glutamate racemase in each cuvette). To determine the amount of L-glutamate present in each sample, the same assay was used with the exception that glutamate racemase was heat-inactivated (100°C, 10 minutes) prior to addition to the assay.

To determine the amount of contamination by glutamate in each sample of N-hydroxyglutamate, Assay A run to completion, was used where D-glutamate was replaced with N-hydroxyglutamate (0.25 mM) and an increased amount of glutamate racemase (3 units) was used.
2.5.11 Enzymatic Reaction of N-Hydroxyglutamate in D₂O

L-N-Hydroxyglutamate (2 mg, 0.012 mmol) was dissolved in 0.25 M deuterated potassium phosphate buffer, pD 8, with 0.2 mM dithiothreitol. Glutamate racemase (125 units), thoroughly exchanged with the same buffer, was added. The progress of the reaction was monitored by \(^1\)H NMR at 37°C using a 400 MHz Bruker WH-400 NMR spectrometer. The analogous experiment with D-N-hydroxyglutamate used 45 units glutamate racemase.

2.5.12 Correlating the Loss of Inhibition with the Loss of D-N-Hydroxyglutamate

D-N-Hydroxyglutamate (1.2 mM) in 0.45 mL of 50 mM Trien-HCl buffer, pH 8, with 0.02 mM DTT and 14.5 units glutamate racemase was incubated at 30°C for 12 minutes (conditions under which 75% of D-N-hydroxyglutamate is converted to α-ketoglutarate). A 0.20 mL aliquot was removed and the reaction was quenched by adjusting the pH to 2 with concentrated hydrochloric acid (3 μL). The denatured enzyme was removed by filtration through a Millipore Ultrafree-4 centrifugal filtration device (10 000 MWCO). To a 50 μL aliquot of the filtrate was added 50 μL 0.5 M Trien-HCl buffer, pH 8 (final pH approximately 7 by pH indicator paper). The sample was assayed for the presence of a racemase inhibitor using Assay A with 0.3 mM D-glutamate. A control sample lacking racemase was similarly prepared and analyzed.
Chapter Three

Alternate Substrates as Probes of the Glutamate Racemase Active Site
3.1 Introduction

3.1.1 Catalytic Cysteine Residues in Enzyme Mechanisms

Experiments on cofactor-independent amino acid racemases and epimerases have indicated that cysteine residues are instrumental in the inversion of stereocentres. Proline racemase, diaminopimelate epimerase and glutamate racemase all require two essential cysteine residues which act as general acid/base catalysts in the deprotonation/reprotonation mechanism. Although the catalytic importance of the cysteine residues in these enzymes is well established, it should be noted that it is rather unusual for cysteines to serve as general acid/base catalysts as opposed to their more typical role as nucleophiles. Many examples of enzymes which utilize cysteine residues as nucleophiles are known. The cysteine protease, papain, for instance, employs a cysteine thiolate to catalyze the cleavage of a peptide bond by forming a thioester intermediate with the peptide at the cleavage site (Figure 3.1) (Stryer, 1988a). Cysteine thiolates also play a vital role in fatty acid biosynthesis catalyzed by the multi-enzyme fatty acid synthase complex in which synthesis proceeds by successive translocations of an elongating fatty acyl chain between a cysteine residue and a coenzyme carrier molecule bearing a thiolate (Stryer, 1988b).

Figure 3.1 The mechanism of peptide bond cleavage by cysteine proteases. The R and R' groups represent the C-terminal and N-terminal peptides, respectively.
Recent reports have suggested that cysteine residues may be involved as general base catalysts in the mechanism of the two enzymes, UDP-GlcNAc enolpyruvyl transferase (Skarzynski, *et al.*, 1998) and 3-methylaspartase (Pollard, *et al.*, 1999). Such examples illustrate the diverse roles which may be assumed by cysteine residues and suggest that cysteines may be employed as general acid/base residues in other enzymes. Racemases and epimerases, however, remain the only well documented examples of enzymes in which cysteine residues are involved in this rather unusual role as general acid/base catalysts.

3.1.2 The Roles of the General Acid/Base Catalysts

In all well documented cases, racemases and epimerases that employ a deprotonation/reprotonation mechanism operate via a two base mechanism as described in Chapter One. A proton is removed from one face of the substrate by an active site general base catalyst and is replaced on the opposite face by a different general acid catalyst. The general acid/base residues may be two different amino acids as in mandelate racemase, a metal-dependent racemase which uses a lysine and histidine residue to invert the enantiomers of mandelate (Powers, *et al.*, 1991). Alternatively, they may be two of the same amino acids as in the cofactor independent enzymes, proline racemase (Rudnick and Abeles, 1975) glutamate racemase (Tanner, *et al.*, 1993) and DAP epimerase (Cirilli, *et al.*, 1998) where the general acid/base residues are two active site cysteines.

Glutamate racemase from *Lactobacillus fermenti* has two cysteine residues, Cys73 and Cys184, whose roles in the deprotonation of the glutamate enantiomers have previously been examined in experiments performed by Knowles and coworkers (Tanner, *et al.*, 1993). The two Cys → Ala mutant enzymes, C73A and C184A, were constructed and found to lack glutamate
racemase activity. However, because the second cysteine residue is still intact in each mutant, these enzymes were able to catalyze a “one base-requiring” reaction. *Threo*-3-chloroglutamate undergoes an enzyme catalyzed elimination of HCl to produce α-ketoglutarate (Figure 3.2 (a)). This reaction requires the participation of only one cysteine residue permitting its use as a “probe” to determine on which enantiomer each cysteine residue acts. When cell extracts containing C184A were incubated with racemic *threo*-3-chloroglutamate, one-half of the *threo*-3-chloroglutamate was converted to α-ketoglutarate. Following the addition of glutamic-oxalacetic transaminase, the remaining 3-chloroglutamate was converted to α-ketoglutarate. Glutamic-oxalacetic transaminase is believed to react only with the (2S,3S)-3-chloroglutamate enantiomer based on the observation that (R)-glutamate and (R)-aspartate are neither substrates nor inhibitors of this enzyme (Jenkins, *et. al*, 1959). The experiment suggests that C184A is capable of catalyzing the elimination of HCl only from (2R,3R)-3-chloroglutamate (Figure 3.2 (b)). Cys184 is therefore the residue responsible for abstracting the hydrogen at C-2 with (S)-configuration. When the same experiment was performed using C73A, again only one-half of the racemic 3-chloroglutamate was consumed, however the addition of glutamic-oxalacetic transaminase resulted in no further conversion of 3-chloroglutamate to α-ketoglutarate. This suggests C73A abstracts a proton at C-2 bearing the (R)-configuration. It is reasonable to assume that the roles of each of the cysteine residues in the 3-chloroglutamate reaction mirrors the roles in the glutamate racemization reaction, thus Cys73 is the residue which abstracts the α-proton from (R)-glutamate (which is equivalent to D-glutamate) and Cys184 removes the α-proton from (S)-glutamate (L-glutamate) in a two-base mechanism.
Figure 3.2 (a) Glutamate racemase catalyzed conversion of threo-3-chloroglutamate to α-ketoglutarate. (b) The reaction of racemic threo-3-chloroglutamate with glutamate racemase mutants, C73A and C184A. GOT is glutamic-oxalacetic transaminase.
3.1.3 N-Hydroxyglutamate as a Probe of the Glutamate Racemase Reaction

A potential problem encountered when one uses alternate substrates to probe the roles of enzymatic residues is that these substrates may bind in the active site in an orientation different from that of the natural substrate. Furthermore, the assignments for the roles of Cys73 and Cys184 of L. fermenti glutamate racemase are based on the assumption that glutamic-oxalacetic transaminase catalyzes the conversion of only the (2S,3S)-3-chloroglutamate diastereomer. It is therefore necessary to confirm the conclusions derived from these experiments since they may not accurately reflect the roles of the catalytic residues in the natural enzymatic reaction.

In Chapter Two, N-hydroxyglutamate was introduced as an alternate substrate for glutamate racemase. We recognized the potential to use this substrate in a series of experiments which would provide additional evidence to establish the roles of the cysteine residues of glutamate racemase. By analogy to threo-3-chloroglutamate, N-hydroxyglutamate is also a one base-requiring substrate since the initial dehydration step of a particular enantiomer of N-hydroxyglutamate by wild type glutamate racemase requires the participation of only a single cysteine residue (Figure 3.3; X=Y=S). Subsequent hydrolysis of the imine intermediate occurs spontaneously to liberate free α-ketoglutarate and ammonia.

![Figure 3.3 Glutamate racemase catalyzed dehydration of N-hydroxyglutamate followed by spontaneous hydrolysis to liberate free α-ketoglutarate. (Wild type glutamate racemase, X=Y=S; Cys→Ser glutamate racemase mutant, X=O, Y=S or X=S, Y=O).](image-url)
The kinetic parameters governing the conversion of a particular enantiomer of N-hydroxyglutamate to α-ketoglutarate may be modified by replacing the cysteine residue responsible for the initial deprotonation event with a serine residue by site-directed mutagenesis (Figure 3.3; X=O, Y=S). The weakly basic serine hydroxyl moiety would continue to abstract the α-proton of the N-hydroxyglutamate enantiomer, however catalytic efficiency would be compromised relative to the wild type enzyme since this residue is not as efficient in catalyzing the dehydration step. The same mutant would catalyze the turnover of the opposite enantiomer of N-hydroxyglutamate with approximately equal efficiency as the wild type enzyme since deprotonation in this direction would involve the intact cysteine residue (Figure 3.3; X=S, Y=O). By comparing the kinetic values obtained with each of the serine mutants, C73S and C184S of L. fermenti glutamate racemase, to the kinetic values obtained using wild type enzyme, it should be possible to establish which cysteine residue of wild type glutamate racemase is involved in the deprotonation of each enantiomer of N-hydroxyglutamate. Presumably, the same assignment would hold for the racemization of glutamate by wild type enzyme.

The Cys→Ser mutants of glutamate racemase also provide a unique opportunity to examine the effects of mutating a thiol acid/base catalyst to an alcohol. The replacement of a cysteine thiol, with a $pK_a$ of ~10, by a serine hydroxyl, with a $pK_a$ of ~16, should impair the ability of the enzyme to efficiently catalyze the inversion of the enantiomers of glutamate due to the lower relative concentration of the serine alkoxide at neutral pH. A serine alkoxide, however, is a more effective Brønsted base than a cysteine thiolate thus it is reasonable to expect that the Ser mutant should retain racemase activity. The activity of the mutant enzyme therefore depends on both the concentrations of the thiolate and alkoxide anions at neutral pH and on the relative rate of deprotonation by the two anions. The effect of the substitution may be studied by
determining the kinetic parameters of the Cys→Ser mutants as well as the dependence of the activity of the mutants on pH.

3.2 Results and Discussion

3.2.1 Preparation of Cys→Ser Mutants of Glutamate Racemase

The *L. fermenti* gene for glutamate racemase (*murl*) has previously been cloned into the BamHI site of the ampicillin resistant pUC18 vector (Gallo and Knowles, 1993a) (Figure 3.4) with loss of one of the BamHI sites at an insert-vector junction. The resulting construct, pKG3, exhibits high overexpression of wild type glutamate racemase despite the absence of a strong promoter in the pUC18 vector. This suggests that a *Lactobacillus* promoter precedes the racemase gene in the expression construct and that this promoter functions in *E. coli* host strains. The promoter is likely found in the non-coding regions which flank *murl* in the construct. Overexpression of gene product is also due in part to the fact that pUC18 is a high copy number vector. The copy number is regulated by the protein Rop, which down-regulates plasmid replication. Replication requires a small piece of RNA which acts as a primer for DNA synthesis. This primer, however, may form a stable complex with a complementary RNA strand in an interaction mediated by Rop. The RNA primer in its duplex form is unable to fulfill its role as a primer of DNA replication, thus plasmid replication is inhibited and copy number decreases (Tomizawa, 1984; Tomizawa and Itoh, 1981). The *rop* gene in pUC18, however, is incomplete, thus bacteria transformed with this vector maintain a high copy number of the plasmid.

The Cys → Ser mutants of glutamate racemase were prepared by introducing the appropriate base pair substitution in the *murl* gene of pKG3 using the recombinant circle...
Figure 3.4 Site map of pKG3. The 2686 base pair plasmid is numbered at appropriate positions to indicate the position of several genetic elements. The lacZ gene in pUC18 is found at 146 - 469, however the gene has been disrupted by insertion of murl. Amp^r is located at 1626 - 2486. DNA replication of these genes proceeds in the same direction as indicated for murl. The murl gene was inserted in the BamHI restriction site and is flanked by short portions of non-coding DNA. The relative positions of the mutagenic primers are indicated.

polymerase chain reaction (RCPCR) method of Jones and Winistorfer (1992) (Figure 3.5). In this technique, the plasmid is linearized in two separate reactions by treating the DNA with a restriction enzyme which cuts the circular DNA at a single, unique restriction site. The polymerase chain reaction is used to amplify the linearized plasmid DNA between two primers, one of which encodes for the desired mutation. Amplified DNA from the two reactions are combined and heat denatured. Because of the overlap between the two ends of the DNA, the complementary portions between the denatured DNA fragments anneal. The annealed DNA is introduced into a bacterial host where the DNA is repaired to produce a complete circular DNA plasmid bearing the desired mutation. The primary advantage of this method is that
In vivo repair

Figure 3.5 Site-directed mutagenesis by the recombinant circle-polymerase chain reaction (RCPCR) method. A mutation is introduced into the gene (black arrow) by amplifying the DNA between the two primers (short arrow), one of which encodes for the desired mutation (triangle on arrow). The double stranded amplified DNA is combined and denatured to allow complementary portions between the DNA to anneal. Following repair in a bacterial host, the circularized DNA plasmid is obtained bearing the appropriate mutation (triangle).
contamination by plasmids encoding for wild type protein is minimized since the template DNA which encodes this protein has been linearized. Linearized DNA transforms bacteria ~100-1000 less efficiently than circular DNA (Cohen, *et. al.*, 1972; Conley and Saunders, 1984) thus providing an initial selection against plasmids expressing wild type protein.

### 3.2.2 Screening for Mutant Activity

A common problem encountered with many mutagenic techniques is that bacterial cells transformed with the products of the PCR reaction are often also transformed with the wild type template DNA plasmid. Furthermore, it is possible that certain plasmids bear an undesired mutation as well as the desired mutation due to misincorporation of bases during DNA synthesis by the DNA polymerase. *Taq* DNA polymerase, which was used in the amplification of plasmid DNA, introduces misincorporations at a rate of about $2.1 \times 10^{-4}$ errors/base. At this rate, undesired second site mutations are likely. A screening technique is necessary to distinguish between wild type DNA plasmids or plasmids bearing a second undesired mutation within the *murL* gene from those with the single base substitution which encodes for the Cys → Ser substitution.

An approach often used to distinguish between wild type DNA plasmids and mutant plasmids is to screen the DNA for a readily detected site introduced by mutagenesis. This method requires the use of properly designed mutagenic primers such that a unique restriction site is either introduced or removed from the plasmid DNA by a silent mutation that does not affect the protein sequence. Only plasmids derived from PCR amplification products have the desired base substitutions, including the modified restriction site, which can be detected by treating the plasmid with the appropriate restriction enzyme. This technique, however, cannot
distinguish between mutant DNA plasmids bearing the correct mutation from those which have additional second site mutations incorporated during amplification.

A more effective approach is to screen for mutants by measuring the level of enzyme activity. One might expect the serine mutants would retain a low level of racemase activity since the serine hydroxyl may still be able to catalyze the deprotonation of the enantiomers of glutamate. This provides a method to screen for mutant enzymes by measuring the activity of the crude protein mixture derived from lysed bacteria transformed with RCPCR plasmid DNA. Cultures displaying high levels of activity equal to a control sample of wild type glutamate racemase are suspected to be wild type contaminants. Plasmid DNA is therefore isolated from intact bacteria which display <10% of wild type activity and the gene is fully sequenced to confirm the presence of only the single desired mutation. This method requires that all bacterial cultures overexpress wild type and mutant glutamate racemases equally to allow a direct comparison of the level of racemase activity between samples.

The latter screening method was used to select plasmids pSG01 encoding for the C73S mutant of glutamate racemase and pSG02 encoding for the C184S mutant enzyme. Enzyme activity was determined by a stopped enzyme assay which quantifies the amount of L-glutamate formed following incubation of the crude protein mixture with D-glutamate. Crude protein mixtures derived from E. coli DH5α transformed with either pSG01 or pSG02 each exhibited ~1% - 10% the activity of a sample of crude wild type enzyme. The glutamate racemase gene of each plasmid was fully sequenced to confirm the presence of the single mutation encoding for the Cys → Ser substitution. The mutant proteins were purified from E. coli DH5α transformed with the appropriate DNA plasmid and analyzed by both SDS-polyacrylamide gel electrophoresis to establish the purity of the enzyme sample (Figure 3.6) and by electrospray ionization mass spectrometry to confirm that the mass of each protein was consistent with that expected from the
amino acid sequence (Expected mass = 28 299 Da (MH\(^+\)); Observed mass C73S = 28 303 ± 4 Da, C184S = 28 303 ± 4 Da).

Plasmid DNA isolated from several bacterial cultures with very low levels of racemase activity, much less than that observed from the gene products of pSG01 and pSG02, were also sequenced. In each of these, at least one undesired base pair substitution was found in the gene sequence.

### 3.2.3 Kinetic Parameters for C73S and C184S

The kinetic parameters for each of the mutants, C73S and C184S, were measured in both the D → L and L → D reaction directions using a known circular dichroism (CD) assay (Gallo and Knowles, 1993a). All aliphatic L-amino acids display a strong positive Cotton effect in the
far UV attributed to the $n \rightarrow \pi^*$ transition associated with the $\alpha$-carboxylate chromophore (Jorgensen, 1971). For L-glutamate the signal is centred at 203 nm when the amino acid is in the zwitterionic form (Katzin and Gulyas, 1968).

The observed CD signal at a particular wavelength, or more precisely the ellipticity $\theta_\lambda$ reported in degrees, is often expressed as the molar ellipticity $[\theta]_\lambda$ with units of deg-cm$^2$-dmol$^{-1}$. The relationship between the measured ellipticity and the molar ellipticity is given by,

$$[\theta]_\lambda = \frac{100 \theta_\lambda}{l \cdot C}$$

where $l$ is the path length in cm, $C$ is the concentration in mol/L and the unitless factor of 100 is a scaling factor. The molar ellipticity at 210 nm for each glutamate enantiomer was found to be 2310 deg-cm$^2$-dmol$^{-1}$ for L-glutamate and -2310 deg-cm$^2$-dmol$^{-1}$ for D-glutamate.

Several limitations are imposed on the sensitivity of this assay as is apparent by the $\sim$20% error in the kinetic values summarized in Table 3.1. At low substrate concentrations, the observed CD signal at 210 nm is very weak such that noise contributions become more significant. The weak signal imposes a lower limit of $\sim$0.5 mM substrate which may be monitored by this technique, an amount greater than the $K_m$ value for wild type enzyme and approaching the $K_m$ values for the mutants. The limited range in substrate concentration results in a large error in the kinetic values, particularly on the $K_m$ determination. At shorter wavelengths up to 203 nm the CD signal becomes slightly stronger ($[\theta]_{204} = 3100$ deg-cm$^2$-dmol$^{-1}$ (Gallo and Knowles, 1993a)), however the favourable increase in signal intensity is offset by an increase in noise observed at shorter wavelengths.

Despite the insensitivity of the assay, we were able to measure the kinetic parameters for the C73S and C184S mutants. The kinetic constants were measured under the same conditions
<table>
<thead>
<tr>
<th></th>
<th>wild type$^a$</th>
<th>C73S</th>
<th>C184S</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>L-Glu</td>
<td>D-Glu</td>
<td>L-Glu</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>69</td>
<td>68</td>
<td>0.19 ± 0.01$^b$</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>0.33</td>
<td>0.26</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$)</td>
<td>2.1 x 10$^5$</td>
<td>2.6 x 10$^5$</td>
<td>(1.6 ± 0.4) x 10$^2$</td>
</tr>
</tbody>
</table>

$^a$Data taken from Gallo and Knowles (1993a). $^b$Errors expressed as standard error of the mean ($n = 3$). $^c$n = 4.
as were used in the earlier determination of the kinetic constants for wild type glutamate racemase (Gallo and Knowles, 1993a). The assay mixture consists of varying concentrations of either D- or L-glutamate in 10 mM potassium phosphate buffer, pH 8, with 0.2 mM DTT. Following the addition of racemase to initiate the reaction, the first 10% of the reaction is monitored by observing the linear change in ellipticity. The rate, \( v \), is obtained from the slope, \( \frac{\Delta \theta}{\Delta t} \), by the equation,

\[
v = 0.5 \cdot \frac{\Delta \theta}{\Delta t} \cdot \frac{1 \text{ mM}}{2.31 \text{ mdeg}}
\]

where the factor of 0.5 accounts for the formation of the opposite enantiomer of glutamate for each turnover, which exhibits an equal but opposite ellipticity. The effective molar ellipticity of 2.31 mdeg/mM accounts for the fact that a cuvette with a 1 mm pathlength was used in all the experiments.

The phosphate buffer used in the study was found to be a competitive inhibitor of both mutant enzymes, with a \( K_i \) of 5.5 mM for C73S and 24 mM for C184S. The wild type enzyme exhibited no significant inhibition at phosphate concentrations up to 30 mM. Since the wild type enzyme is not inhibited by phosphate, a direct interaction likely exists in the mutant enzyme between the serine hydroxyl and phosphate. Due to competitive inhibition by phosphate buffer, all \( K_m \) values were corrected using the following equation,

\[
K_m = \frac{K_{m, \text{app}}}{(1 + \frac{[I]}{K_i})}
\]

The serine mutation decreases the \( k_{cat} \) values by about 1 to 2 orders of magnitude relative to wild type enzyme while the \( K_m \) increases by \(~10\)-fold for both mutants, indicating that the serine substitution affects the ability of the enzyme to bind the enantiomers of glutamate (Table
3.1. For each mutant, the $k_{cat}/K_m$ values are equal within error in both reaction directions, a condition which satisfies the Haldane equation for a racemase

$$K_{eq} = \frac{(k_{cat}/K_m)_L}{(k_{cat}/K_m)_R}.$$ 

The overall effect of mutating the cysteine residues to serine is to reduce the $k_{cat}/K_m$ value by a factor of $1.2 \times 10^3$ for C73S and $6 \times 10^2$ for C184S. If a thiolate/alkoxide is necessary for the reaction to occur, it may be expected that a $10^6$-fold decrease should be observed for the serine mutant since at a given pH the relative concentration of the serine alkoxide is $10^6$-fold less than that of the cysteine thiolate (based on a six unit difference in $pK_a$’s between the thiol ($pK_a \sim 10$) and the hydroxyl ($pK_a \sim 16$)). However, the greater relative reactivity of the alkoxide anion could offset this effect. The relationship between the effectiveness of a general base catalyst and the $pK_a$ is expressed by the Brønsted catalysis law which may be written as,

$$\Delta p_{k_{cat}} = \beta \cdot \Delta p_{K_a}.$$ 

Assuming a Brønsted $\beta$ value of 0.50 for the deprotonation of glutamate, a $10^3$-fold greater relative reactivity for the serine alkoxide is expected. The two effects, the relative concentration and the relative reactivity of the anions, would result in a $10^3$-fold greater reactivity for the thiolate anion (based on the ratio between the two effects, $10^6/10^3$), as is observed.

3.2.4 pH Dependence of $k_{cat}/K_m$

The pH dependence on $k_{cat}/K_m$ was determined for the mutant and wild type enzymes in the D-glutamate to L-glutamate direction using the coupled enzyme assay described in Chapter Two which couples the formation of L-glutamate by glutamate racemase to the reduction of $p$-iodonitrotetrazolium violet. These pH titrations reflect the ionization of the free enzyme or free substrate and are often dominated by titration of the key catalytic residues. The activity of the enzyme was measured at various pH values by monitoring the racemization of D-glutamate at a
single low substrate concentration (0.3 mM). Under such conditions, the reaction is governed by a first order process described by the equation, rate = k_{obs}[S] where k_{obs} = \frac{k_{cat}}{K_m} [E]_0, allowing the $k_{cat}/K_m$ value to be determined in a single reaction by fitting the data to a first order rate equation. This eliminates the need to correct for variation in the extinction coefficient exhibited by the chromophore, p-iodonitrotetrazolium violet, at different pH values which would be necessary if the $k_{cat}$ and $K_m$ values were obtained at each pH value from the initial velocity of a series of reactions at varying substrate concentrations.

The coupled enzyme assay uses L-glutamate dehydrogenase as one of the coupling enzymes. This enzyme, however, exhibits low activity at pH<7 (Olson and Anfinsen, 1953) limiting the effective pH range which may be examined using this assay. Although the CD assay is also available, it is not a viable alternative since the insensitivity of the assay does not allow the reaction to be effectively monitored at low concentrations of glutamate. The $k_{cat}/K_m$ values were therefore determined over a narrow pH range of 7-9 using the coupled enzyme assay. At each pH value, checks were made to ensure the reaction was fully coupled.

The $k_{cat}/K_m$ pH profiles of wild type and mutant glutamate racemase are shown in Figure 3.7. The pH profile of wild type glutamate racemase is a narrow bell shaped curve, as is expected for an enzyme which has two catalytic cysteine residues of similar pK_a values, one of which must be in the deprotonated thiolate form to serve as a general base and the other in the protonated thiol form to serve as a general acid. The pH profile for C73S suggests the pH at which optimal rate is achieved has shifted to a pH value greater than 9. In the D-glutamate to L-glutamate reaction direction, the serine alkoxide is presumably the residue responsible for deprotonation of the D-enantiomer based on work with Cys → Ala glutamate racemase mutants with threo-3-chloroglutamate. At more basic pH values, the higher relative concentration of the serine alkoxide is reflected in the sharp increase in rate. In the C184S mutant, the cysteine
thiolate is expected to be the residue responsible for the deprotonation of D-glutamate. Overall, very little dependence on pH is observed over the pH range examined with this mutant. It therefore appears that the C184S mutant is in the correct protonation state over this narrow pH range.

Based on the logic applied to the interpretation of the pH profile in the D-glutamate to L-glutamate reaction direction, it is expected that a different pH profile should be obtained in the L-glutamate to D-glutamate reaction direction. With C73S, for example, the cysteine residue would be involved in the deprotonation of L-glutamate and the pH profile should resemble a wide bell-shaped curve with the ascending limb reflecting the cysteine thiol ionization and the descending limb reflecting the serine hydroxyl ionization. This would seemingly violate the Haldane equation which predicts a ratio of unity between the $k_{cat}/K_m$ values in the two reaction directions. In fact, the Haldane relationship reflects both the catalytic interconversion of substrate to product and the interconversion of the two enzyme forms, a process which may be rate limiting (Cleland, 1963). Typically, enzyme interconversion is not considered in a $k_{cat}/K_m$ pH dependence since this process is assumed to be rapid. In fact, the oversaturation kinetics performed on wild type glutamate racemase presented in Chapter One suggest that interconversion of the two enzyme forms at pH 8 is significantly faster than the racemization rate having very little effect on $k_{cat}/K_m$. Enzyme interconversion, however, is a pH-dependent process and at the pH extremes there may be a significant contribution by enzyme interconversion. In addition, the mutation of Cys to Ser may slow the interconversion dramatically so that it is potentially rate determining in the mutant enzyme. Slow enzyme interconversion has previously been noted for proline racemase and fumarase (Rose, et. al, 1992; Fisher, et. al. 1986). It would be interesting to examine the $k_{cat}/K_m$ pH dependence in the reverse direction to see if the pH profiles are indeed different in the L-glutamate to D-glutamate reaction direction.
Figure 3.7 Partial pH rate profile for the racemization of D-glutamate by (a) wild type glutamate racemase and (b) the C73S and C184S mutants.
3.2.5 Kinetic Parameters for the Cys → Ser Mutants with N-Hydroxyglutamate

The enzyme catalyzed elimination of water from N-hydroxyglutamate presumably requires the participation of only one of the catalytic cysteine residues. Replacement of the cysteine thiol by a serine hydroxyl should impair the turnover of only one N-hydroxyglutamate enantiomer (Figure 3.3; X=O, Y=S). This affect should be readily observed upon comparing the second order rate constants for elimination of water from both N-hydroxyglutamate enantiomers by the mutant and wild type enzymes.

The specificity constants were determined for each enantiomer of N-hydroxyglutamate with each mutant enzyme using the enzyme assay described in Chapter Two. This assay couples the formation of α-ketoglutarate from N-hydroxyglutamate to the loss of NADH with L-glutamate dehydrogenase. The racemase catalyzed formation of α-ketoglutarate from N-hydroxyglutamate is monitored by following the reaction to completion at a single low concentration of substrate. The second order rate constant is determined by fitting a first order exponential decay equation, rate = k_{obs}[S] where k_{obs} = \frac{k_{cat}}{K_m} [E]_0, to the data. By using a low concentration of substrate, problems due to inhibition of L-glutamate dehydrogenase by N-hydroxyglutamate are minimized.

The results in Table 3.2 clearly demonstrate that the C73S catalyzed elimination of water from D-N-hydroxyglutamate is impaired by the mutation suggesting that in this mutant, the serine residue is responsible for deprotonation of D-N-hydroxyglutamate (Figure 3.3, X=O, Y=S). The turnover of the L-enantiomer by C73S, however, is more efficient than turnover by wild type enzyme. This may be due to a better capacity of the mutant to accommodate the increased bulk in the substrate. The opposite result is observed with the C184S mutant. The conversion of L-N-hydroxyglutamate is impaired while that of D-N-hydroxyglutamate is improved relative to wild
Table 3.2 Kinetic Constants for N-Hydroxyglutamate Reactions

<table>
<thead>
<tr>
<th></th>
<th>D-N-Hydroxyglutamate</th>
<th>L-N-Hydroxyglutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>3.2 x 10^3</td>
<td>30</td>
</tr>
<tr>
<td>C73S</td>
<td>58</td>
<td>160</td>
</tr>
<tr>
<td>C184S</td>
<td>4.6 x 10^3</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

*Errors in $k_{cat}/K_m$ are approximately ±10% and are due to the error in enzyme concentration.

type enzyme. This suggests that in the C184S mutant, the serine residue is responsible for the deprotonation of L-N-hydroxyglutamate.

These results support the notion that each cysteine residue specifically deprotonates a single enantiomer and that in the wild type enzyme, Cys73 is responsible for deprotonation at C-2 of D-glutamate while Cys184 deprotonates L-glutamate. This corroborates the assignment made in earlier studies on Cys $\rightarrow$ Ala mutants of glutamate racemase and the effect the mutation had on the elimination of HCl from theo-3-chloroglutamate.

### 3.3 The Utility of Alternate Substrates

The experiments described in this chapter demonstrate the utility of alternate substrates in defining the roles of catalytic residues in an enzyme reaction. Such an approach has been used on related enzymes and may present a general method for probing an enzymatic reaction by a combination of site directed mutagenesis and use of available enzyme substrates or inhibitors.
The roles of the catalytic residues of mandelate racemase have been studied by experiments similar to those described in this chapter. Mandelate racemase facilitates the interconversion of (R)- and (S)-mandelate by chelating the substrate with a metal ion to polarize the negative charge of the carbonyl group thereby lowering the pK\textsubscript{a} of the \(\alpha\)-proton and potentially stabilizing the carbanion intermediate (Figure 3.8) (Buckingham, et. al., 1967). On the basis of high resolution crystal structures of mandelate racemase, His297 and Lys166 are presumed to be the active site general acid/base catalysts in the enzyme (Neidhart et. al., 1991; Landro et. al., 1994). Studies on the H297N mutant of mandelate racemase demonstrate that although the enzyme is completely inactive as a racemase, it retains the ability to stereospecifically exchange the \(\alpha\)-proton of (S)-mandelate with a solvent derived deuteron (Landro et. al., 1991). This observation is consistent with deprotonation of (S)-mandelate by a polyprotic residue, such as Lys166, to afford a transiently stable intermediate that is sufficiently long-lived to allow stereospecific delivery of a deuteron by rotation of the lysine C-N bond.

The crystal structure of the adduct that forms on treatment of mandelate racemase with (R)-\(\alpha\)-phenylglycidate (31) reveals a covalent adduct formed by nucleophilic attack of the \(\varepsilon\)-amino group of Lys166 on the epoxide ring (Landro et. al., 1994). Since (R)-\(\alpha\)-phenylglycidate and (S)-mandelate are configurationally equivalent, the stereospecific alkylation of Lys166 by the inhibitor provides strong support that this residue is responsible for proton transfers on the (S) face of mandelate.

Figure 3.8 The reaction catalyzed by mandelate racemase
Further studies examined the ability of both the H297N and K166R mutants of mandelate racemase to catalyze the stereospecific elimination of bromide from (R,S)-p-(bromomethyl)-mandelate (32). The H297N mutant eliminates bromide only from (S)-p-(bromomethyl)-mandelate (Landro et al., 1991) while K166R specifically catalyzes the elimination of bromide ion from (R)-p-(bromomethyl)mandelate (Kallarakal et al., 1995). These observations are consistent with deprotonation of (S)-mandelate by Lys166, and of (R)-mandelate by His297.
3.4 Experimental Methods

3.4.1 Materials

All reagents were purchased from Sigma or Aldrich. Ultrapure potassium phosphate and ultrapure potassium hydroxide were purchased from Aldrich. Wild type glutamate racemase was purified from *E. coli* DH5α bearing the pKG3 as described (Gallo and Knowles, 1993a). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

3.4.2 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:

01 GGTCGTGGCCCTCCAATACGGCGA
02 GATTATGGGCTCCACCCACTTCC
03 TCGCCGTATTGGAGGCCACGACC
04 GGAAGTGGGTGAGGGCACCAGACC
05 CGACGAGCGTGCACACCACGATGCC
06 GCAGAGCGAGGTATGAGGGCGGTGC
Primers 01, 02, 03 and 04 are complementary to the glutamate racemase gene. Primers 05 and 06 are complementary to the pUC18 vector (Appendix B and C). Primers 01 and 03 encode for the C73S mutation and primers 02 and 04 encode for the C184S mutation. Underlined regions encode for the Cys → Ser mutation.

3.4.3 Site-Directed Mutagenesis

Mutants were prepared by the recombinant circle PCR technique described by Jones and Winistorfer (1992). The pKG3 vector bearing the glutamate racemase gene *murl* was linearized in two separate reactions with either EcoR1 (Promega) or HindIII (Gibco-BRL). Each of these restriction enzymes cuts pKG3 at a single unique restriction site. Two PCR amplification reactions were performed on the EcoR1 linearized DNA: reaction 1 (C73S) contained primers 01 and 06 and reaction 2 (C184S) contained primers 02 and 06. The HindIII linearized DNA was amplified using the following primer pairs: reaction 3 (C73S) used primers 03 and 05 and reaction 4 (C184S) used primers 04 and 05. PCR reactions (100 μL) contained 2 ng of linearized DNA, 25 pmol of each primer, 0.4 mM of each dNTP, 3 mM MgCl₂ and 2.5 U Taq DNA polymerase (Gibco-BRL) in the supplied PCR buffer. DNA was amplified by an initial denaturation step at 94°C for 3 minutes followed by 16 cycles of denaturation at 94°C for 1.5 minutes, annealing at 50°C for 2 minutes and extension at 72°C for 4 minutes. After the last cycle, an additional 4 minutes at 72°C ensured complete extension. Recombinant DNA plasmids were obtained by combining the products of reactions 1 and 3 (C73S) and separately combining reactions 2 and 4 (C184S). The combined PCR products were denatured at 94°C for 3 minutes and annealed at 50°C for 2 hours. CaCl₂-competent *E. coli* DH5α bacterial cells were transformed with the recombinant DNA plasmid. 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.

### 3.4.4 Screening for Mutant Activity

A 2.5 mL aliquot was removed from the 5 mL overnight bacterial cultures. Plasmid DNA was isolated from the sample using a Wizard Miniprep DNA Purification System (Promega). The remaining culture was used to determine the relative degree of enzyme activity expressed in each sample. Bacterial cells were pelleted and redissolved in 30 mM triethanolamine-HCl buffer, pH 7.5, containing 10% glycerol, 0.2 mM DTT, 1 mg/L pepstatin, 1 mg/L aprotinin and 1 mM phenylmethlysulfonyl fluoride (Stock solutions of 5 mg/mL pepstatin, 5 mg/mL aprotinin and 100 mM phenylmethlysulfonyl fluoride were all prepared in ethanol). Cells were lysed by one pass through a French pressure cell (SLM Aminco) at 20 000 psi. The lysate was centrifuged at 3000 rpm for 5 minutes. A small sample was removed for SDS-polyacrylamide gel electrophoresis to compare the relative overexpression levels of glutamate racemase between the samples. The crude protein concentration of each sample was determined by the method of Bradford (1976). Protein samples were appropriately diluted with the above buffer to ensure equal amounts of crude protein in all samples. A previously described stopped enzyme assay was used to evaluate the amount of glutamate racemase activity in each sample (Gallo and Knowles, 1993a). Protein samples were incubated in an equal volume of 50 mM potassium phosphate buffer, pH 7.5, containing 10% glycerol, 1 mg/L bovine serine albumin, 2 mM DTT and 150 mM D-glutamate in a total volume of 400 µL. The samples were incubated at 37°C for one hour then heated at 100°C for 10 minutes. Denatured protein was removed by centrifugation at 13 000 rpm in a microcentrifuge for 5 minutes. The amount of L-glutamate in a
50 µL sample was quantitated in an assay containing 10 mM NAD⁺ and 30 units L-glutamate dehydrogenase in 50 mM Tris-HCl buffer, pH 9.5, (1 mL total volume) and monitoring the change in A₃₄₀ nm at 30°C. The extinction coefficient of NADH was taken as 6220 M⁻¹·cm⁻¹. A control sample of wild type glutamate racemase was similarly treated.

Plasmid DNA from samples which displayed ~1-10% of wild type glutamate racemase activity were sequenced on a PE Applied Biosystems Model 373 Automated DNA Sequencer (Nucleic Acid and Protein Service Unit, UBC) using primer 07 (CCACCACGGCAACCACCA) and primer 08 (CCGGGTGCTCTTAAAGG). Two plasmids with the correct mutation, pSG01 (C73S) and pSG02 (C184S) were obtained.

### 3.4.5 Purification of Mutant Glutamate Racemase

*E. coli* DH5α transformed with either pSG01 or pSG02 were grown in 1 L of LB broth supplemented with 50 µg/mL ampicillin. The cells were harvested by centrifugation, resuspended in Buffer A (30 mM triethanolamine-HCl buffer, pH 7.5, with 10% glycerol, 2 mM DTT, 1 mg/L pepstatin, 1 mg/L aprotinin and 1 mM phenylmethylsulfonyl fluoride) and lysed by two passes through a French pressure cell at 20 000 psi. Following ultracentrifugation at 30 000 rpm for 45 minutes, the supernatant was loaded onto a 50 mL diethylaminoethyl cellulose (Whatman) column that had been pre-equilibrated with Buffer A. The column was washed with the same buffer (200 mL) and protein was eluted with Buffer A containing 200 mM NaCl. Protein-containing fractions were desalted by repeated concentration and dilution with buffer A using a Millipore Ultrafree-15 centrifugal filtration device (MWCO 10 000). Protein was further purified using a Waters 625 HPLC system on a Waters AP-1 Protein-Pak Q 8 HR column (10 x 100 mm) that had been pre-equilibrated with Buffer B (50 mM triethanolamine-HCl buffer, pH
8, containing 10% glycerol and 2 mM DTT). After introduction of the protein, the column was washed with Buffer B (10 mL) and eluted with a linear gradient of NaCl (0 M → 0.4 M) over 30 minutes at a flow rate of 1 mL/min. Eluted protein was assayed for glutamate racemase activity using Assay A described in Chapter Two. Racemase-containing fractions were combined, desalted and reapplied to a Waters AP-1 Protein-Pak Q 8HR HPLC column that had been pre-equilibrated with Buffer C (50 mM triethanolamine-HCl buffer, pH 7, containing 10% glycerol and 0.2 mM DTT). The column was washed with Buffer C and protein was eluted with a linear gradient of NaCl (0 M → 0.4 M) over 30 minutes at a flow rate of 1 mL/min. Racemase-containing fractions were pooled and concentrated as above. The purity of the racemases was assessed by SDS-polyacrylamide gel electrophoresis on a 12% acrylamide gel using a Bio-Rad Mini-PROTEIN II electrophoresis system. Protein bands were visualized by staining with Coomassie Brilliant Blue. The molecular weight of the pure racemase was determined by electrospray ionization mass spectrometry on a PE-SCIEX API 300 MS operated by Shouming He in the laboratory of Dr. Stephen Withers, Department of Chemistry, UBC.

3.4.6 Circular Dichroism Assay for Glutamate Racemase Activity

A previously described circular dichroism assay was used to determine the initial velocity of the reaction catalyzed by glutamate racemase in both the D-glutamate → L-glutamate direction and in the L-glutamate → D-glutamate direction (Gallo and Knowles, 1993a). The ellipticity of a solution of glutamate in 10 mM potassium phosphate buffer, pH 8, with 0.2 mM DTT equilibrated at 30°C was monitored in a 1 mm cuvette at 210 nm on a JASCO 710 Circular Dichroism Spectrophotometer following the addition of enzyme. The molar ellipticity of L-glutamate was taken as 2310 deg·cm²·dmol⁻¹.
3.4.7 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 and mutant enzyme was determined by following the reaction to completion at a low substrate concentration (0.03 mM) using Assay A described in Chapter Two with the exception that 50 mM Tris-HCl buffer (pH 7 - 9) replaced the triethanolamine-HCl buffer. An assay was initially performed to establish that the reaction was coupled over the pH range examined in this study by adding an additional equivalent of glutamate racemase to a reaction mixture which was being monitored at 500 nm and observing a doubling of the rate. In order that the reaction was coupled at all pHs, the amount of diaphorase was increased to 4 units and the amount of L-glutamate dehydrogenase was increased to 67.5 units. Data were fitted to a first order rate equation using the graphics program, GraFit (Erithacus Software Ltd., 1994).

3.4.8 Measurement of Second Order Rate Constants for N-Hydroxyglutamate

The specificity constants for conversion of D- and L-N-hydroxyglutamate into α-ketoglutarate by the mutant enzymes were measured by following the progress of the reaction to completion at a low N-hydroxyglutamate concentration (0.02 mM) using Assay B described in Chapter Two. The reaction was initiated by the addition of 0.35 mg C73S to monitor the loss of either D- or L-N-hydroxyglutamate. The loss of D-N-hydroxyglutamate by C184S required 0.1 mg of enzyme while L-N-hydroxyglutamate required >1 mg of enzyme to observe a decrease in $A_{340}$. A first order rate equation was fit to the data using the graphics program, GraFit.
Chapter Four

Isotope Effects on Cys→Ser Mutants of Glutamate Racemase:
The Roles of the Catalytic Acid/Base Residues
4.1 Introduction

4.1.1 Kinetic Isotope Effects in Enzymatic Reactions

Many methods used to study enzymatic mechanisms rely on alternate substrates to examine the nature of the reaction. A significant drawback accompanying this approach is that little is known about the changes which such substrates induce on an enzymatic reaction. A technique that most nearly avoids the inherent problems associated with alternate substrates uses isotopes as probes of a reaction mechanism. Isotopic substitution has no effect on the qualitative chemical reactivity of the substrate, but it often has a readily measured quantitative effect on the reaction rate.

One type of isotopic substitution experiment involves measuring the primary kinetic isotope effect on an enzymatic reaction (Cleland et al., 1977). A primary isotope effect is observed when a C-H bond that is broken in a rate limiting step of a reaction is replaced with a C-D bond. The greater mass of deuterium contributes to a weaker vibrational energy and a lower zero-point energy for the C-D bond which results in a greater energy difference between the initial state and the transition state in the C-D bond breaking step. The simplest model describing the magnitude of primary kinetic isotope effects predicts $\frac{k^H}{k^D}$ values of 6.4, where $k^H$ is the rate constant for hydrogen abstraction and $k^D$ is the rate constant for deuterium abstraction (Kresge, 1977). This value is based on the loss of zero-point energy that accompanies the conversion of vibrational motion into translational motion as the C-H bond breaks. The zero-point energy of a typical C-H stretching vibration with $v=3000$ cm$^{-1}$ is 4.3 kcal/mol; deuterium substitution reduces the frequency to 2200 cm$^{-1}$ and the zero-point energy to 3.2 kcal/mol. The difference, $\Delta E_0 = 1.1$ kcal/mol, gives $\frac{k^H}{k^D} = \exp(\Delta E_0/RT)=6.4$, which is often quoted as the theoretical limit for an intrinsic primary kinetic isotope effect. The intrinsic isotope effect is the full effect originating from the single isotopically sensitive step, exclusive of all interference
from isotopically insensitive steps. With 2800 cm\(^{-1}\) and 3300 cm\(^{-1}\) as reasonable limits for the C-H vibration, the model predicts \(^1H\)\(^/D\)\(^k\) values ranging from 5 to 8. This model, however, does not account for the well-known wide variation in the magnitude of isotope effects. In enzymatic reactions, the observation of small hydrogen isotope effects often results from the multistep nature of these reactions with isotopically insensitive steps masking the contribution by the isotopically sensitive step (Kresge, 1977). Large isotope effects may be a consequence of quantum mechanical tunnelling. In systems where the wavelength of the proton is comparable to the width of the energy barrier, molecules with insufficient energy to surmount the energy barrier may "leak" through the energy barrier and show primary kinetic isotope effects >20 (Klinman, 1978).

Kinetic isotope effects may be considered with respect to their relationship to the fractionation factors of the kinetically important species involved in the reaction (Schowen, 1977). The fractionation factor \(\phi\) for a site R is defined as the equilibrium constant for the isotopic exchange equilibrium,

\[
\text{RH} + 1/2 \text{D}_2\text{O} \rightleftharpoons \text{RD} + 1/2 \text{H}_2\text{O} \quad \text{(Scheme 4.1)}
\]

where the deuterium fractionation factor for R is

\[
\phi = \frac{[\text{RD}][\text{H}_2\text{O}]^*}{[\text{RH}][\text{D}_2\text{O}]^*}. \quad \text{(Equation 4.1)}
\]

The relationship between the fractionation factors and the isotope effect is readily appreciated from the following scheme.

\[
\text{RH} \xrightarrow[H^k]{H} [\text{TH}] \\
\text{RD} \xrightarrow[D^k]{D} [\text{TD}] \\
\]

The reactant RH with one exchangeable hydrogen, proceeds through the transition state TH, also
with one exchangeable hydrogen, in a process with rate constant $^\text{H}k$. If the system were introduced into deuterated water, exchange would occur in the reactant state to generate RD with an exchange equilibrium constant of $^\text{R}\phi$, the reactant isotopic fractionation factor, and in the transition state to generate TD with an equilibrium constant of $^\text{T}\phi$. The process would now proceed with a rate constant $^\text{D}k$. Because Scheme 4.2 consists of a closed cycle,

\[
^\text{H}k \cdot ^\text{T}\phi = ^\text{R}\phi \cdot ^\text{D}k
\]

and $^\text{D}k = \frac{^\text{R}\phi}{^\text{T}\phi}$  

(Equation 4.2)

4.1.2 $V_{\text{max}}$ and $V_{\text{max}}/K_m$ Isotope Effects

The glutamate racemase reaction may be described by the following reaction scheme

\[
E_1 + S \xrightleftharpoons[k_1]{k} E_1S \xrightarrow[k_2]{k_2} E_2P \xrightarrow[k_3]{k_3} E_2 + P
\]

(Scheme 4.3)

where the interconversion of the two free enzyme forms, $E_1$ and $E_2$, which differ only in their protonation states, is assumed to be rapid. Product release is considered to be irreversible since the concentration of product is assumed to be zero during initial velocity measurements. The kinetic expressions describing the mechanism of Scheme 4.3 are (Cleland, 1975) (Appendix D):

\[
V_{\text{max}} = \frac{k_2k_3[E_1]}{k_2 + k_2 + k_3}
\]

(Equation 4.3)

\[
\frac{V_{\text{max}}}{K_m} = \frac{k_1k_2k_3[E_1]}{k_1k_2 + k_1k_3 + k_2k_3}
\]

(Equation 4.4)

By definition, $V_{\text{max}}$ represents the rate at saturating substrate concentrations, which
eliminates the binding component \((k_1 \text{ and } k_{-1})\), leaving the catalytic and product release components \((k_2, k_{-2} \text{ and } k_3)\). Assuming product dissociation is much faster than the chemical steps \((i.e. k_3 > k_2 \text{ and } k_{-2})\), \(V_{\text{max}}\) is dependent only on the catalytic steps \((k_2 \text{ and } k_{-2})\). The \(V_{\text{max}}\) isotope effect therefore reduces to the intrinsic isotope effect, \(H_{k_2}/D_{k_2}\). The \(V_{\text{max}}/K_{\text{m}}\) value represents all rate constants up to and including the first irreversible step, which in the example described above includes the binding, catalysis and product release components. Isotope effects measured on \(V_{\text{max}}\) and \(V_{\text{max}}/K_{\text{m}}\) are therefore dependent on different components of a reaction mechanism and provide different information about the reaction mechanism.

4.1.3 \(V_{\text{max}}\) and \(V_{\text{max}}/K_{\text{m}}\) Isotope Effects for Glutamate Racemase

The \(V_{\text{max}}\) and \(V_{\text{max}}/K_{\text{m}}\) primary isotope effects have previously been measured for wild type \(L. \text{ fermenti}\) glutamate racemase (Table 4.1) (Tanner et al., 1993). Isotope effects were observed in both reaction directions suggesting deprotonation of either enantiomer is not the cleanly rate-determining step. The isotopically insensitive step, reprotonation of the intermediate, partially masks the intrinsic isotope effect on substrate deprotonation. Since approximately equal isotope effects were observed in both directions, the reaction profile describing the reaction is expected to be symmetrical, as demonstrated in Figure 4.1, where neither the deprotonation step nor the reprotonation step are cleanly rate determining.

The dependence of \(V_{\text{max}}\) and \(V_{\text{max}}/K_{\text{m}}\) on the individual rate constants is based on the assumption that interconversion of the two enzyme forms is rapid (Cleland, 1975). If enzyme interconversion is not rapid however, the \(V_{\text{max}}\) isotope effect, in addition to representing the intrinsic isotope effect \(H_{k_2}/D_{k_2}\), should also include a term representing enzyme interconversion. At low concentrations of substrate, the \(V_{\text{max}}/K_{\text{m}}\) isotope effect is presumably independent of the
Table 4.1 \( V_{\text{max}} \) and \( V_{\text{max}}/K_m \) Isotope Effects for wild type \( L. \text{fermenti} \) Glutamate Racemase

<table>
<thead>
<tr>
<th>Reaction Direction</th>
<th>( V_{\text{max}} ) Isotope Effect</th>
<th>( V_{\text{max}}/K_m ) Isotope Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glu ( \rightarrow ) L-Glu</td>
<td>3.1 ± 0.5</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>L-Glu ( \rightarrow ) D-Glu</td>
<td>2.2 ± 0.4</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.4</td>
<td>1.36</td>
</tr>
</tbody>
</table>

\(^{a}\)Data taken from Tanner et. al. (1993).

enzyme interconversion process since sufficient enzyme should be in the correct ionization state at pH 8 (This is based on the assumption that the \( pK_a \) of the cysteine general base is \( \sim 8 \). The presence of the negatively charged thiolate may influence the \( pK_a \) of the general acid catalyst which would have a slightly higher \( pK_a \)). If enzyme interconversion were significantly rate determining, the \( V_{\text{max}} \) isotope effect would be expected to be partially masked by the isotopically insensitive interconversion of the two enzyme forms and the value for the isotope effect would approach unity. Since the isotope effect values on \( V_{\text{max}} \) are the same within the limits of error as

---

\[ \text{Figure 4.1} \quad \text{Partial reaction profile describing the deprotonation/reprotonation of bound glutamate by wild type glutamate racemase.} \]
the values on \( \frac{V_{\text{max}}}{K_m} \), an additional slow step such as enzyme interconversion is not significantly rate limiting for wild type glutamate racemase at pH 8. This confirms the conclusion drawn from results obtained by the oversaturation experiment described in Chapter One (Gallo et. al., 1993b).

### 4.1.4 Isotope Effects on Cys→Ser Mutants of Glutamate Racemase

The mechanism of the glutamate racemase reaction may be further explored with experiments that utilize isotopically labelled substrates and mutant glutamate racemases. The ultimate objective of these experiments is to gain insight into the role of the cysteine residues in the inversion of the enantiomers of glutamate. A Cys → Ser mutation should affect the isotope effects in both reaction directions and may provide evidence to establish which cysteine residue is responsible for the deprotonation of each glutamate enantiomer.

It was reported in Chapter Three that the serine mutants of glutamate racemase retain a low level of racemase activity. The effect of the mutation may be described graphically by an asymmetry in the reaction profile where the dominant energy barrier for the serine mutant is the step involving deprotonation/reprotonation by the weakly basic serine side chain (Figure 4.2 (b) and (c)). The mutation-induced asymmetry in the reaction profile may be readily verified by measuring the primary isotope effects in the two reaction directions.

Earlier work on glutamate racemase mutants with threo-3-chloroglutamate (Tanner et. al., 1993) and \( N \)-hydroxyglutamate enantiomers suggest Cys73 is responsible for deprotonation of D-glutamate and Cys184 is responsible for deprotonation of L-glutamate. The expected reaction profile for the C73S catalyzed reaction should therefore reflect the greater difficulty for abstraction of the \( \alpha \)-proton of D-glutamate by a serine residue relative to abstraction by the
cysteine residue of wild type glutamate racemase. For the C73S catalyzed reaction, deprotonation of D-glutamate is expected to be more cleanly rate determining than it is in the wild type reaction suggesting the reaction profile shown in Figure 4.2 (b) applies. The intrinsic isotope effect associated with deprotonation of D-glutamate should be partially unmasked and a larger isotope effect should be observed in the D → L reaction direction. In the reverse reaction direction, the isotopically sensitive step, the deprotonation of L-glutamate by the Cys184 residue, is less cleanly rate determining since it is partially masked by the subsequent reprotonation step and a smaller isotope effect is expected in the L → D direction. The reverse situation is expected with the C184S mutant (Figure 4.2 (c)). The serine residue is responsible for deprotonation of L-glutamate and the intrinsic isotope effect is partially unmasked in the L → D direction. A larger primary kinetic isotope effect is expected in the L → D reaction direction relative to wild type enzyme and a smaller isotope effect is expected in the D → L direction.
4.1.5 Competitive Deuterium Washout Experiment

A convenient method for determining the $V_{max}/K_m$ isotope effect for the glutamate racemase reaction has been described by Tanner et. al. (1993) as modified from previous work by Albery and Knowles (Albery and Knowles, 1987; Fisher et. al., 1986). This method, based on a circular dichroism spectrophotometric assay, monitors the ellipticity of a system which maintains near equilibrium concentrations of substrate and product. A typical experiment consists of a solution of equimolar amounts of L-[2-$^2$H]glutamate and D-[2-$^1$H]glutamate in H$_2$O. The solution has a measured ellipticity of zero since the deuterium label does not significantly affect the molar ellipticity of the labelled substrate. Following the addition of glutamate racemase, a small imbalance in the equilibrium concentration occurs. This is because the L-enantiomer is produced from unlabelled D-glutamate more rapidly than the D-enantiomer is produced from deuterated L-glutamate due to the kinetic isotope effect. This perturbation is
readily detected as a transiently positive change in the ellipticity of the solution (Figure 4.3). As the racemization reaction proceeds, the deuterium label is washed out into solution, the equilibrium condition is once again re-established and the ellipticity returns to zero.

In a series of equations derived by Albery and Knowles (1987), a relationship has been established between the maximum deviation in the ellipticity reading, $\Delta \theta_{\text{max}}$ (expressed as a mole fraction of the total glutamate, $\lambda_{\text{max}}$) and the $V_{\text{max}}/K_{m}$ primary kinetic isotope effect. Under the experimental conditions, the $V_{\text{max}}/K_{m}$ isotope effect may be described by the composite rate equation, $\frac{h}{k_{1,2,3}}$, (Appendix D) where,

$$2\lambda_{\text{max}} = (R-2)R^{R/1-R} \quad \text{(Equation 4.5)}$$

$$\lambda_{\text{max}} = \frac{\Delta \theta_{\text{max}}}{2.31 \text{ mdeg/mM} \cdot [\text{Glu}]_{\text{tot}}^{105}} \quad \text{(Equation 4.6)}$$

and

$$R = 2 \cdot \frac{h}{k_{1,2,3}} \quad \text{(Equation 4.6)}$$

The primary advantage of this method is that the $V_{\text{max}}/K_{m}$ isotope effect is determined in a single reaction thereby reducing the error inherent to experiments which compare the results between different experimental runs. Furthermore, because the result is independent of enzyme concentration the activity of the enzyme may change over the course of the experiment without affecting the result. Errors are primarily introduced into the experiment in the concentrations of the enantiomers of glutamate and by the noise in the ellipticity at $\Delta \theta_{\text{max}}$. 
4.2 Results and Discussion

4.2.1 Preparation of Deuterated Glutamate Enantiomers

Pure enantiomers of D and L-[2-2H]glutamate were prepared by the method described by Tanner et al. (1993). Racemic deuterated glutamate was prepared using glutamate racemase in D2O to specifically introduce deuterium label at the C-2 position of glutamate. Following N-acetylation with acetic anhydride, the enantiomers were resolved using porcine kidney acylase I which specifically deacetylates the L-enantiomer providing L-[2-2H]glutamate. Acid hydrolysis of the remaining N-acetyl-D-[2-2H]glutamate yielded pure D-[2-2H]glutamate. Both enantiomers were obtained in >98% enantiomeric excess and with a deuterium content of >97%.

4.2.2 $V_{\text{max}}/K_m$ Isotope Effects for Mutant Glutamate Racemase

The $V_{\text{max}}/K_m$ isotope effects for C73S and C184S were determined in both directions using the competitive deuterium washout experiment. With either mutant, the ellipticity of the solution shows a perturbation to more positive values when the L-enantiomer is deuterated (Figure 4.4 (a) shows the data obtained with C184S). This is a result of the transient accumulation of L-glutamate due to the isotope effect associated with its racemization. From the maximum perturbation in ellipticity ($\Delta \theta_{\text{max}}$) observed with C184S, an isotope effect of $4.8 \pm 0.02$ for the racemization of L-glutamate is calculated (Table 4.2). This is a significant increase from the wild type isotope effect of $2.5 \pm 0.1$ measured in the same reaction direction and suggests that chemistry is more cleanly rate determining relative to wild type enzyme. This result is consistent with the reaction profile shown in Figure 4.2 (c) and suggests that in this mutant...
the serine hydroxyl is responsible for the deprotonation of L-glutamate. The same argument would require that a decreased isotope effect should be observed in the D → L direction since in this reaction direction the isotopically sensitive step is masked by the subsequent rate determining reprotonation of the intermediate. When the D-enantiomer is deuterated, a smaller, negative change in the ellipticity is observed (Figure 4.4 (b)). The corresponding isotope effect calculated for C184S in the D → L direction is 2.3 ± 0.1 compared to the isotope effect for

<table>
<thead>
<tr>
<th>Reaction Direction</th>
<th>Wild type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C73S</th>
<th>C184S</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glu → L-Glu</td>
<td>3.4 ± 0.1</td>
<td>5.1 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>L-Glu → D-Glu</td>
<td>2.5 ± 0.1</td>
<td>1.85 ± 0.01</td>
<td>4.80 ± 0.02</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.36 ± 0.07</td>
<td>2.76 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.48 ± 0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data from Gallo and Knowles (1993a). <sup>b</sup>Errors are standard errors of the mean (n = 3). <sup>c</sup>Errors are standard errors of the ratio.
wild type enzyme of 3.4 ± 0.1. This result is again consistent with the reaction profile shown in Figure 4.2 (c). Cys184 is therefore the residue responsible for deprotonation of L-glutamate.

With the C73S mutant, the kinetic isotope effect in the D → L direction increases to 5.1± 0.3 relative to the wild type value of 3.4± 0.1, whereas the isotope effect in the reverse direction is smaller than the wild type value (1.85 vs. 2.5). This result is consistent with the reaction profile shown in Figure 4.2 (b) and suggests Cys73 is responsible for deprotonation of D-glutamate. The results agree with the assignments based on studies with N-hydroxyglutamate and on earlier work with threo-3-chloroglutamate. The different values measured for the isotope effects in the two reaction directions also provides evidence for a stepwise two base mechanism proceeding through a carbanion intermediate. If the reaction were to follow a concerted mechanism, there would be no difference expected between the $V_{max}/K_m$ isotope effects in the forward and reverse reaction directions.

It could be argued that the observed increase in the isotope effect on mutating a cysteine residue to a serine residue may be due to the difference in ground state fractionation factors between the cysteine thiol ($\phi = 0.40$) and the serine hydroxyl ($\phi = 1.0$) (Schowen, 1978). Transition state fractionation factors are typically between 0.12 and 0.4. From the relationship between isotope effects and fractionation factors (Equation 4.2), a larger isotope effect would be predicted for the reaction catalyzed by the serine hydroxyl. Although this may contribute to the observed increase in the isotope effect in one reaction direction, it fails to account for the observed decrease in the isotope effect in the reverse reaction direction. The result is most reasonably explained by an asymmetry in the reaction profile as described.
4.2.3 Double Competitive Deuterium Washout Experiment

The results obtained with the competitive deuterium washout experiment may be cross-checked using the double competitive deuterium washout experiment (Fisher et. al., 1986, Tanner et. al., 1993). This experiment is similar to the competitive deuterium washout experiment with the exception that equimolar amounts of each deuterated enantiomer are incubated with glutamate racemase in H₂O. The larger isotope effect associated with the turnover of a particular glutamate enantiomer by the mutant racemase results in a transient accumulation of that enantiomer in solution. Analogous to the competitive deuterium washout, the ellipticity of the equimolar solution initially at zero deviates either towards more positive or more negative ellipticity values. The direction of the perturbation is dictated by the larger isotope effect associated with the turnover of a particular glutamate enantiomer. As the deuterium label continues to washout from glutamate, equilibrium is re-established and the ellipticity returns to zero. Figure 4.5 demonstrates the result obtained on incubating equimolar amounts of deuterated D,L-glutamate with C184S. The larger isotope effect in the L → D

![Figure 4.5](image_url)
direction exhibited by this mutant results in a temporary accumulation of L-glutamate and a positive deviation in the ellipticity from zero.

The maximum deviation in ellipticity from zero, $\Delta \theta_{\text{max}}$ (expressed as the mole fraction of the total glutamate, $\lambda_{\text{max}}$), is related to the ratio of the $V_{\text{max}}/K_m$ isotope effects in the two reaction directions ($\xi$) for a particular enzyme by the following equation,

$$2\lambda_{\text{max}} = (1 - \xi) \xi^{(1 - \xi)}.$$ 

The ratio between the isotope effect in the $D \rightarrow L$ direction to the isotope effect in the $L \rightarrow D$ direction was found to be $2.80 \pm 0.01$ for C73S and $0.52 \pm 0.03$ for C184S. These values agree very well with the ratios of the $V_{\text{max}}/K_m$ isotope effects determined by the competitive deuterium washout experiment (Table 4.2), providing further confirmation for the earlier experimental values.

4.3 Further Studies: $V_{\text{max}}$ Isotope Effects of Glutamate Racemase Mutants

Although the $V_{\text{max}}$ isotope effects on C73S and C184S were not measured, further studies may examine the difference between the isotope effects on $V_{\text{max}}$ to the isotope effects on $V_{\text{max}}/K_m$. It would be interesting to see how the two values compare since this may provide some insight into the contribution of isotopically insensitive steps on the observed isotope effects. If the $V_{\text{max}}$ isotope effects were significantly different from isotope effects on $V_{\text{max}}/K_m$, an additional step such as enzyme interconversion may be rate limiting for the mutant enzymes. It is known that enzyme interconversion for wild type glutamate racemase is not rate determining at pH 8 (Gallo et. al., 1993). It would be interesting to observe the influence of the Cys$\rightarrow$Ser
mutation on the rate of enzyme interconversion in each reaction direction.

4.4 Mutation-Induced Perturbations in the Reaction Profiles of Various Racemases

4.4.1 Alanine Racemase

Very recent studies have examined the roles of the catalytic general acid/base residues of alanine racemase from *Bacillus stearothermophilus* by methods which observe the influence on the kinetic isotope effects following mutation of each of the two catalytic residues. The enzyme is believed to use Lys39 and Tyr265 in a two base PLP-dependent mechanism to invert the enantiomers of alanine. This assignment is based on X-ray crystal structures which demonstrate that these two residues are appropriately positioned to be the general acid/base catalysts (Shaw *et. al.*, 1997; Stamper *et. al.*, 1998; Morollo *et. al.*, 1999). Studies on K39A mutants of *B. stearothermophilus* alanine racemase demonstrate that the mutant is completely inactive despite the presence of PLP in the free aldehyde form in the enzyme active site (Watababe, 1999). By employing the chemical rescue method of Toney and Kirsch (1989) and replacing the function of the Lys residue lost through mutagenesis by the addition of 0.2 M methylamine, the authors were able to restore the catalytic activity of alanine racemase in both the L→D and D→L reaction directions to about 0.1% of wild type activity.

Earlier experiments on alanine racemase by Faraci and Walsh demonstrated that there was virtually no isotope effect on $V_{max}$ for abstraction of the α-proton from both D- and L-alanine by *B. stearothermophilus* alanine racemase (Faraci and Walsh, 1988). The failure to observe an isotope effect suggests that in the wild type enzyme, abstraction of the α-proton is not the rate determining step. The most likely rate-limiting step is the transaldimination reaction between the bound substrate and the PLP cofactor (Figure 4.6(a)). A strong $V_{max}$ isotope effect of 5.4 was
Figure 4.6 Partial reaction profiles of alanine racemization by (a) B. stearothermophilus alanine racemase; (b) the methylamine-rescued K39A mutant of alanine racemase and (c) the R219 mutants of alanine racemase. The relative height of the energy barrier in Figure (a) describing the trans-aldimination reaction between the amino moiety of the substrate and the PLP cofactor is unknown (Faraci and Walsh, 1988).

observed, however, for the B. stearothermophilus methylamine-assisted racemization of D-alanine to L-alanine by K39A. No isotope effect was observed in the $L \rightarrow D$ direction. This supports the notion that the chemical rescue was acting on a step involving deprotonation and that this step was primarily rate determining in the rescued mutant (Figure 4.6 (b)).

Solvent isotope effects were also measured for the rescued mutant racemase in each reaction direction. A solvent isotope compares the rate of a reaction run in $H_2O$ versus $D_2O$, expressed as a ratio of the two rates, $k_{H_2O}/k_{D_2O}$. Active site functional groups may become labelled by exchange with $D_2O$ giving rise to a solvent isotope effect when a deuterium label is transferred during the course of an enzymatic reaction. A solvent isotope effect was observed only in the $L \rightarrow D$ direction suggesting methylamine takes on the role of Lys39; the conjugate acid of methylamine serves as a general acid in the reprotonation of the intermediate to form D-alanine in the $L \rightarrow D$ direction and as a base in the deprotonation of D-alanine. The asymmetry in the kinetic isotope effects suggests a second general base residue, presumably Tyr265, specifically acts on the L-alanyl-PLP aldimine intermediate.
4.4.2 Mandelate Racemase

Mandelate racemase isolated from the bacterium *Pseudomonas putida* is another enzyme in which a mutation-induced perturbation in the reaction profile has been used to examine the roles of the catalytic residues. Mandelate racemase uses His297 and Lys166 to interconvert the enantiomers of mandelate (Landro *et. al.*, 1994). The K166R mutant retains a low level of racemase activity and is postulated to have an asymmetric reaction profile as demonstrated in Figure 4.7 (Kallarakal *et. al.*, 1995). This assumption is based on the fact that the K166R mutant exhibits a substrate isotope effect on $V_{\text{max}}$ of 3.6 ± 0.2 in the (S) $\rightarrow$ (R) direction which is unchanged from the isotope effect on wild type enzyme of 3.56 ± 0.12 (Whitman *et. al.*, 1985). In the (R) $\rightarrow$ (S) direction, however, a negligible substrate isotope effect is measured (1.1 ± 0.1) compared to the wild type isotope effect of 3.2 ± 0.11. This is a result of the subsequent reprotonation step which is primarily rate determining. The observed effect on the substrate isotope effect confirms the predicted asymmetry in the reaction coordinate for the K166R mutant of mandelate racemase as compared to the symmetric reaction profile for wild type mandelate racemase and suggests that the role of deprotonation of (S)-mandelate belongs to Lys166. A
similar experiment could not be performed on His297 mutants since the H297N mutant exhibited no racemase activity.

4.5 Summary

Isotopic substitution provides a unique opportunity to study the dynamic nature of a natural enzymatic reaction, avoiding the problems and limitations inherent to experiments which use alternate substrates to probe an enzyme mechanism. This technique is not without its own limitations, however. Mutation of the relevant enzymatic residues must not destroy enzyme activity and the isotopically sensitive step must be at least partially rate determining. Provided these conditions are met, this method has the potential to determine the importance of not only those residues directly involved in an enzymatic reaction but conceivably of other important residues which serve an essential though indirect role.
4.6 Experimental Methods

4.6.1 Materials

Ultrapure potassium phosphate (99.99%) and ultrapure potassium hydroxide (99.99%) were purchased from Aldrich. All remaining reagents were purchased from Sigma or Aldrich. The mutant glutamate racemase enzymes, C73S and C184S, were purified from *Escherichia coli* DH5α bearing the pSG01 or pSG02 plasmids, respectively, as described in Chapter Three. D2O was purchased from Cambridge Isotope Laboratories. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as the standard.

D- and L-[2-2H]-Glutamate were prepared and analyzed by the method of Tanner *et al.* (1993). N-Acetyl-D,L-[2-2H]glutamate was prepared by racemizing glutamate in D2O using glutamate racemase followed by N-acetylation using acetic anhydride and 6 M NaOH. The enantiomers of N-acetyl-D,L-[2-2H]glutamate were resolved by treatment with porcine kidney acylase I in D2O to yield L-[2-2H]glutamate. The remaining N-acetyl-D-[2-2H]glutamate was hydrolyzed with 5 M HCl to isolate D-[2-2H]glutamate. The enantiomeric purity of each sample was evaluated as described. Briefly, the enantiomeric purity of each sample was determined by derivatizing the dimethyl ester of each deuterated glutamate enantiomer with (R)-(+)α-methoxy-α-(trifluoromethyl)phenylacetyl chloride followed by gas chromatographic analysis on a Hewlett-Packard HP-17 GC column. The deuterium content of the derivative was established by comparing the DCI mass spectrum of derivatized deuterated glutamate to the spectrum for derivatized undeuterated glutamate.
4.6.2 Competitive Deuterium Washout Experiment

The competitive deuterium washout experiment was performed as previously described (Tanner et. al., 1993). The CD signal of a 250 μL solution equilibrated at 30°C containing 5 mM deuterated substrate and 5 mM undeuterated enantiomer in 10 mM potassium phosphate buffer, pH 8, with 0.2 mM DTT, was monitored at 210 nm following the addition of 0.07 mg of the appropriate mutant enzyme. The molar ellipticity of L-glutamate at 210 nm was taken as 2310 deg-cm$^2$-dmol.

4.6.3 Double Competitive Deuterium Washout Experiment

The double competitive deuterium washout experiment was performed as previously described (Tanner et. al., 1993). The CD signal of a 250 μL solution containing equal amounts of each deuterated enantiomer (6 mM each for C184S and 2.5 mM each for C73S) in 10 mM potassium phosphate buffer, pH 8, containing 0.2 mM DTT was monitored at 210 nm and 30°C following the addition of 0.07 mg of enzyme.
Chapter Five

Polar Residues in the Active Site
of Glutamate Racemase
5.1 Introduction

Of the hundreds of amino acids that make up a typical enzyme, only a small fraction are
directly involved in executing the catalytic conversion of substrates to products. A simplistic
view of an enzyme may consider that the remaining amino acid residues simply serve as a
scaffold to appropriately position the catalytic residues for participation in the enzyme reaction,
however, this overlooks their often essential, though indirect, participation in an enzymatic
reaction. These residues may be involved in modulating the properties of catalytic residues
through interactions which enhance the catalytic efficiency of the enzyme, in stabilizing the
transition states along a reaction coordinate by interacting with these high energy species or in
binding the substrate to position it in the active site proximal to catalytic residues. A thorough
understanding of an enzyme mechanism addresses the importance of these participating residues
in the reaction. To this end, the roles of amino acid residues in the active site of glutamate
racemase have been examined to understand how such residues may enhance the efficiency of
the enzymatic reaction.

Catalysis by glutamate racemase involves deprotonation of a glutamate enantiomer by a
cysteine thiolate (Gallo and Knowles, 1993a). The presence of an unstabilized thiolate under
physiological conditions however is unfavourable since the $pK_a$ of the cysteine side chain is ~10.
This effect is even more pronounced with the serine mutants of glutamate racemase in which an
alkoxide with a $pK_a$ of ~16 is involved in the deprotonation of glutamate. As described in
Chapter Three, the observed $10^3$-fold decrease in the mutant $k_{cat}/K_m$ value relative to wild type
enzyme may be understood if the reaction involves an alkoxide. Furthermore, the dependence on
hydroxide ion concentration observed for the C73S mutant (Figure 3.7) is consistent with a
reaction that proceeds through the basic Ser73 alkoxide ion. It therefore seems likely that the
reaction involves a thiolate in wild type enzyme or an alkoxide in the Cys → Ser mutants and suggests the enzyme has developed a means to stabilize the anionic form of these residues.

A low $pK_a$ is required for the cysteine thiol or serine hydroxyl to be an effective base catalyst under the near-neutral pH conditions at which the enzyme operates. A local polar environment at the enzyme active site may stabilize the anionic form of the general base catalyst by lowering the $pK_a$ of the cysteine side chains. This would allow a cysteine thiolate to act as the general base catalyst and a cysteine thiol to act as a general acid catalyst under physiological conditions. Another plausible mechanism involves stabilization of the cysteine thiolate, or alkoxide in the case of the serine mutants, by either hydrogen bonding to acidic residues at the active site (Figure 5.1) or by electrostatic interaction with a positively charged residue. In the first situation, any protonated group with a reasonable $pK_a$, such as aspartate, glutamate, tyrosine or histidine would be suitable, but in the second situation, only histidine, arginine, lysine and the N-terminus are viable options. An interaction with one of these residues could potentially lower the $pK_a$ of cysteine to a near-neutral value by stabilization of the thiolate anion. Aspartate and glutamate, with solution $pK_a$'s of ~4, would require a less polar active site to stabilize the protonated forms of these residues. Under such conditions, the thiolate would be even less stable, such that considerable stabilization may be provided by the hydrogen bonding interactions.

![Diagram](image)

*Figure 5.1 Deprotonation of glutamate by a cysteine thiolate stabilized by hydrogen bonding interactions with adjacent amino acid residues.*
The importance of a particular active site residue of glutamate racemase may be examined by mutagenesis to investigate the potential stabilizing influence of these residues on the cysteine thiolate. Mutating such a residue should selectively affect the ability of the cysteine residue which it stabilizes to act as a general base in the deprotonation of glutamate. Appropriate amino acid substitutions which alter the hydrogen bonding properties at that position allow the importance of the hydrogen bonding interaction to be studied with minimal effect on the enzyme structure. For example, glutamate and aspartate residues involved in hydrogen bonding stabilization interactions may be replaced by their less acidic isosteres, glutamine and asparagine (Figure 5.5). These residues may retain the ability to hydrogen bond with the thiol/thiolate, however if substantial transfer of a proton from the carboxylic acid group of glutamate or aspartate toward the thiolate is important to catalysis, this substitution is expected to reduce $k_{cat}$ since the $pK_a$ of a carboxamide group is $\sim 15$. The less acidic isostere would prevent significant transfer of a proton thereby affording less stabilization of the thiolate. In the absence of efficient stabilization of the cysteine thiolate, the $pK_a$ of cysteine in the active site should increase to $\sim 10$, the $pK_a$ for this residue in solution. The substitution, however, should not disrupt the active site geometry since the carboxamide functional group could retain hydrogen bonding interactions with other functional groups and ordered water molecules in the active site. Similar results are expected with appropriate amino acid substitutions of histidine, tyrosine, arginine or lysine residues.

An approach which uses an alternate substrate such as $N$-hydroxy glutamate may be used to examine the effect of an amino acid substitution on the catalytic conversion of the enantiomers of $N$-hydroxy glutamate to $\alpha$-ketoglutarate and ammonia. If mutation of a residue impairs the turnover of a particular $N$-hydroxy glutamate enantiomer, the residue is possibly involved in stabilizing the cysteine in the thiolate form. Technical difficulties, however, make it difficult to
perform such an experiment. Since only the $k_{\text{cat}}/K_m$ values can be measured for $N$-hydroxyglutamate using the available coupled enzyme assay, the result would not indicate if the effect is expressed strictly on $K_m$, which is reasonable if the residue is necessary for binding and proper orientation of the substrate in the active site, or on catalysis, $k_{\text{cat}}$, if the residue is essential for stabilization of a thiolate residue or a combination of both binding and catalysis. This technique also suffers from the inherent problems associated with alternate substrates, in that little is known about the changes induced on an enzymatic reaction by such substrates.

An experiment which avoids the difficulties associated with the use of alternate substrates involves measuring the primary kinetic isotope effects for mutants of glutamate racemase. The reaction profile which describes the reaction catalyzed by the mutant glutamate racemase should be asymmetric if the replaced residue is involved in a stabilizing interaction with the cysteine thiolate (Figure 5.2), similar to the asymmetric energy profile induced by the Cys $\rightarrow$ Ser mutation. The loss of a stabilizing interaction may manifest itself in a reaction step which is more cleanly rate determining. A mutation-induced asymmetry in the reaction profile may be readily verified by the difference in the isotope effects in the two reaction directions.

Active site amino acid residues may also be involved in stabilizing the transition states and intermediates which are encountered along the reaction path. In the glutamate racemase reaction, a residue may act as a general acid catalyst, distinct from the general acid involved in reprotonating the enolate intermediate, by donating a proton towards the carbonyl oxygen of the substrate to neutralize the developing negative charge on the oxygen as the reaction proceeds towards the intermediate (Figure 5.3). It has been proposed that if the $pK_a$s of the general acid catalyst and the enol tautomer of the substrate are matched, significant stabilization of the enolic intermediate relative to the more weakly basic keto tautomer may be achieved by formation of a low barrier hydrogen bond (LBHB) (Gerlt and Gassman, 1993b). A corollary to the stabilization
Figure 5.2 Partial reaction profiles for the racemization of glutamate by (a) wild type glutamate racemase and (b) a mutant enzyme lacking hydrogen bonding stabilization of the cysteine thiolate.
Figure 5.3 General acid catalyzed stabilization of the enolate intermediate.

of the intermediate by this mechanism is that the $pK_a$ of the $\alpha$-proton of the substrate is reduced in the active site from the value measured in solution to enhance the removal of the $\alpha$-proton.

An intermediate-stabilizing interaction has been postulated in the mechanism catalyzed by mandelate racemase from *Pseudomonas putida* (Mitra et al., 1995). The X-ray crystal structure of mandelate racemase with a competitive inhibitor bound in the active site exhibits a hydrogen bond between the carboxylic acid group of Glu317 and the carboxylate of the bound inhibitor (Landro et al., 1994). This residue presumably participates as a general acid catalyst in the concerted general acid/base catalyzed formation of the enol intermediate. Studies on the E317Q mutant of mandelate racemase provide evidence that this residue may be involved in stabilizing the reaction intermediate by hydrogen bonding interactions since loss of the hydrogen donating ability of Glu317 by the E317Q mutation results in a significant $3 \times 10^4$-fold reduction in $k_{cat}/K_m$.

If a similar strong interaction is involved in stabilizing the carbanionic intermediate in the glutamate racemase catalyzed reaction, mutation of this residue should have a significant impact on the $k_{cat}/K_m$ value. Furthermore, the reaction profile describing the reaction should remain relatively symmetric, and one might expect to see an increase in the isotope effects in both reaction directions.
Finally, active site residues involved in binding and positioning the substrate in the active site may similarly be studied by observing the effect that mutation of such residues has on the kinetic parameters of the resulting mutant enzyme and on the energy profile describing the reaction. Loss of such a residue by mutation should have a significant effect on the $K_m$ value. The reaction profile for the mutant may remain symmetrical, however asymmetry is also likely since an enzyme is a chiral molecule with an asymmetric active site. Although the enzyme binds equally to each glutamate enantiomer, mutation of a particular residue may have a greater impact on the binding of only one substrate enantiomer and may result in an asymmetric reaction profile with one step being more cleanly rate determining.

5.2 Results and Discussion

5.2.1 Multiple Sequence Alignment

The first step in the study involves identifying residues which may participate in a stabilizing interaction with the cysteine thiolate. Such residues serve an essential role in the enzyme mechanism and should be conserved over the process of evolution. Since enzymes from different bacterial sources are likely to have originated from a common ancestral protein, a comparison of the amino acid sequence of various isozymes indicates residues which have been highly conserved during evolution. Highly conserved residues often have an essential role in the enzyme mechanism since mutation of this residue destroys enzyme activity. Non-essential
Figure 5.4 Multiple sequence alignment of the 13 isozymes of glutamate racemase. (LACFI =
Lactobacillus fermenti; LACBR = Lactobacillus brevis; PEDPE = Pediococcus pentosaceus;
BACSH = Bacillus sphaericus; STAHA = Staphylococcus haemolyticus; BACSU = Bacillus
subtilis; MYCLE = Mycobacterium leprae; MYCTU = Mycobacterium tuberculosis; ECOLI =
Escherichia coli; HAEIN = Haemophilus influenzae; SYNY3 = Synechocystis sp. strain PCC
6803; AQPYR = Aquifex pyrophilus; HELPY = Helicobacter pylori. Strictly conserved residues
are indicated by (*); residues with similar properties (i.e. polarity, charge, aromaticity) are
indicated by (:); residues not as closely related but share some similarities including topology or
properties are indicated by (.).
Figure 5.4 (continued) Multiple sequence alignment of the 13 isozymes of glutamate racemase.

```plaintext
LACFI
LACBR
PEDPE
BACSH
STAHA
BACSU
MYCLE
MYCTU
ECOLI
HAEIN
SYNY3
AQPYR
HELPY

Figure 5.4 (continued) Multiple sequence alignment of the 13 isozymes of glutamate racemase.

```
amino acid residues may undergo a wide variation of amino acid substitutions with minimal effect on the enzyme activity.

A primary sequence alignment of the 13 distinct isozymes of glutamate racemase using the CLUSTAL W (1.74) alignment algorithm (Thompson et. al., 1994) indicates Asp10, Asp36, Glu152 and His186 of L. fermenti glutamate racemase are strictly conserved across all known glutamate racemase sequences (Figure 5.4). Although other residues are strictly conserved, these residues are of interest since they could potentially stabilize either the reaction intermediate or the cysteine thiolate of wild type glutamate racemase by hydrogen bonding interactions. The two residues D10 and H186 are found in highly conserved regions. This may be used as an indicator of the importance of these residues in the enzyme mechanism since adjacent residues, although perhaps not directly involved in the mechanism, help to appropriately position and modulate the essential amino acid for participation in the enzyme reaction. The region surrounding E152 is somewhat less conserved while that surrounding D36 is not very conserved. These residues serve as a reasonable starting point to examine the importance of stabilizing interactions in the active site of glutamate racemase.

An X-ray crystal structure of glutamate racemase from Aquifex pyrophilus with D-glutamine bound in the active site was made available during the time these experiments were being conducted (Hwang, 1999) although the coordinates have not been released. The residues equivalent to D10, E152 and H186 of the L. fermenti enzyme are conserved in the A. pyrophilus enzyme and are located in the enzyme active site. The residue equivalent to D36 is also conserved in the A. pyrophilus enzyme, however, it is not found in the active site of the enzyme. The X-ray crystal structure of the enzyme is discussed in detail in Section 5.2.6.
Figure 5.5 Isosteric amino acid side chain residues. R = HOOC-CH-NH₂

5.2.2 Preparation of Mutant Enzymes

Each of the four conserved residues were individually substituted with an isosteric amino acid which lacks efficient hydrogen donating character. The glutamate and aspartate residues were replaced with glutamine and asparagine, respectively, while histidine was replaced with asparagine (Figure 5.5). If either of these residues are necessary in stabilization interactions, the mutation should disrupt the efficiency of the racemization reaction catalyzed by glutamate racemase.

The recombinant circle polymerase chain reaction (RCPCR) technique (Jones and Winistorfer, 1992) used to prepare the Cys → Ser mutants of glutamate racemase was used to prepare the four mutant plasmids bearing murJ genes encoding for D10N, D36N, E152Q and H186N. Whereas the earlier mutagenesis protocol used Tag DNA polymerase to amplify pKG3 plasmid DNA, Pwo DNA polymerase was used in this protocol. Pwo DNA polymerase
introduces misincorporations at a rate of $-4 \times 10^{-6}$ errors/base, which is significantly better than the misincorporation rate of Taq DNA polymerase ($-2.1 \times 10^{-4}$). The improved fidelity is a consequence of the 3'-5' exonuclease activity of the Pwo DNA polymerase which proofreads the DNA inserted by the 5'-3' processive DNA polymerase activity. Taq DNA polymerase bears only the 5'-3' processive DNA polymerase activity and cannot correct for misincorporations. The use of Pwo DNA polymerase therefore reduces the number of plasmids which have undesired mutations in the murl sequence.

Bacteria transformed with the RCPCR-derived plasmids were screened for mutant enzyme activity by assaying the level of racemase activity expressed by these cells. This method, which was previously used to screen for Cys $\rightarrow$ Ser mutants of glutamate racemase, compares the level of racemase activity expressed by a bacterial culture transformed with plasmids derived from RCPCR mutagenesis to a control sample transformed with pKG3, the plasmid encoding for wild type glutamate racemase. Bacterial cultures expressing a low level of racemase activity relative to the wild type control are assumed to be mutant enzymes. Fortuitously, the base pair substitution which encodes the D10N mutation also introduces a new EcoR1 restriction site into the plasmid DNA. Treatment with EcoR1 excises a $-450$ base pair fragment between the EcoR1 site derived from the polylinker region of the pUC18 expression construct and the additional EcoR1 site introduced by the D10N-encoding mutation (Figure 5.6). The fragment may be readily detected by separating the DNA fragments using agarose gel electrophoresis. Plasmids which encode for the D10N mutant of glutamate racemase result in two DNA fragments whereas wild type contaminants result in a single fragment of linear DNA.
Figure 5.6 Treatment of the pSG03 plasmid encoding for the D10N mutant of glutamate racemase with EcoR1 results in two fragments approximately 450 and 3600 base pairs in length.

Plasmids were submitted for DNA sequencing to confirm the presence of the single desired mutation in the murI gene. Plasmids encoding for D10N (pSG03), D36N (pSG04), E152Q (pSG05) and H186N (pSG06) were obtained. Each of the mutant proteins were purified from E. coli DH5α transformed with the appropriate DNA plasmid and analyzed by both electrospray ionization mass spectrometry to confirm that the mass of each protein was consistent with that expected from the amino acid sequence (Table 5.1) and by SDS-polyacrylamide gel electrophoresis to establish the purity of the enzyme sample.
Table 5.1 Expected and observed masses of mutant glutamate racemases determined by ESI-MS.

<table>
<thead>
<tr>
<th></th>
<th>Expected Mass MH⁺ (Da)</th>
<th>Observed Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10N</td>
<td>28 315</td>
<td>28 315 ± 4</td>
</tr>
<tr>
<td>D36N</td>
<td>28 315</td>
<td>28 315 ± 4</td>
</tr>
<tr>
<td>E152Q</td>
<td>28 315</td>
<td>28 316 ± 4</td>
</tr>
<tr>
<td>H186N</td>
<td>28 293</td>
<td>28 294 ± 4</td>
</tr>
</tbody>
</table>

5.2.3 Kinetic Parameters for Mutant Enzymes

The kinetic parameters of the four mutant enzymes were measured in both the L → D and D → L directions using the circular dichroism assay described in Chapter Three. These values are compared to those measured for wild type glutamate racemase in Table 5.2. In contrast to the Ser mutants of glutamate racemase, these mutants are not inhibited by phosphate buffer at concentrations up to 50 mM phosphate.

The turnover rate, $k_{\text{cat}}$, is most severely affected by the mutation of D10 and H186. In both the L → D and D → L reaction directions, the $k_{\text{cat}}$ value for the D10N mutant is reduced by a factor of $1 \times 10^3$ relative to wild type enzyme. The H186N mutation reduces the $k_{\text{cat}}$ value by a factor of $1.5 \times 10^3$ in the L → D direction and $7.3 \times 10^2$ in the D → L direction. From this data, it may be concluded that substituting a carboxamide functional group for a carboxylic acid or imidazole functional group at residues 10 and 186 causes a significant reduction in both $k_{\text{cat}}$ and
Table 5.2 Kinetic constants for wild type glutamate racemase and mutant glutamate racemases D10N, D36N, E152Q and H186N

<table>
<thead>
<tr>
<th></th>
<th>L-Glutamate</th>
<th>D-Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$K_m$ (mM)</td>
</tr>
<tr>
<td>Wild Type$^a$</td>
<td>69</td>
<td>0.33</td>
</tr>
<tr>
<td>D10N</td>
<td>0.068 ± 0.004$^b$</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>D36N</td>
<td>20 ± 3</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>E152Q</td>
<td>38 ± 4</td>
<td>5.8 ± 0.5</td>
</tr>
<tr>
<td>H186N</td>
<td>0.045 ± 0.004</td>
<td>1.1 ± 0.3</td>
</tr>
</tbody>
</table>

$^a$Data from Gallo and Knowles (1993a). $^b$Errors expressed as standard error of the mean (n = 3)
Comparatively, the $k_{cat}/K_m$ values for the D36N and E152Q mutants are decreased by a more modest factor of about 2- to 3-fold over the value for wild type enzyme. However, the $K_m$ values measured for the D36N are increased by two order of magnitude relative to wild type, compared to a 3-17 fold increase in the range of $K_m$ values for the other three mutants. The significant effect on the $K_m$ value suggests the D36 residue is important for binding the glutamate substrate. Mutation of the E152 residue has the least effect on the enzymatic efficiency with the second order rate constant reduced by only 32-fold in the $L \rightarrow D$ reaction direction and 43-fold in the $D \rightarrow L$ reaction direction. The $k_{cat}/K_m$ values in the two reaction directions are the same within error for all mutant in the two reaction directions, as predicted by the Haldane equation, except for the H186N mutant. Perhaps interconversion of the two enzyme forms is partially rate determining for this mutant.

5.2.4 $V_{max}/K_m$ Isotope Effects

It was hoped that the $V_{max}/K_m$ isotope effects could be measured using the competitive deuterium washout experiment described in Chapter Four. This method has many advantages over methods which measure the initial velocity of varying concentrations of deuterated substrate, including the independence of the result on enzyme concentration which often contributes to significant error and the ability to determine the isotope effect in a single reaction. Attempts to measure the isotope effects using the competitive deuterium washout experiment, however, proved difficult. The racemization of glutamate by D10N and H186N mutants is severely impaired in both reaction directions such that reaction times approaching three hours are necessary to reach $\Delta \theta_{max}$. Over the course of such long experiments, significant drift is observed in the ellipticity signal. It is possible to shorten the reaction time with the addition of increased
amounts of enzyme, however very large amounts of enzyme are necessary to decrease reaction times to a point where the error introduced by drift is insignificant. At high concentrations of enzyme, the signal-to-noise ratio decreases as incident light is absorbed by the protein in solution. These problems precluded attempts to measure the $V_{\text{max}}/K_m$ isotope effects for D10N and H186N. Kinetic isotope effects on the D36N mutant were not measured since the X-ray crystal structure indicated D36 is not located in the active site.

E152Q racemizes the enantiomers of glutamate efficiently which allows the $V_{\text{max}}/K_m$ isotope effects for this enzyme to be readily measured using the competitive deuterium washout experiment (Table 5.3). The isotope effects were measured in both the $D \rightarrow L$ and $L \rightarrow D$ reaction directions and were found to be larger than the corresponding isotope effects for the wild type enzyme measured in the same direction by a factor of 1.2. The ratio between the isotope effects is $1.40 \pm 0.02$ for E152Q which is the same as the ratio calculated for wild type enzyme ($1.36 \pm 0.07$). The data suggests the reaction profile is largely symmetrical, much like that seen for wild type enzyme, however the deprotonation of either enantiomer is more cleanly rate determining in the E152Q mutant relative to wild type enzyme (Figure 5.7 (b)).

The increase in the kinetic isotope effect in the two reaction directions suggests the residue may be involved in stabilizing the intermediate by acting as a general acid, similar to the postulated involvement of the E317 residue in mandelate racemase. In mandelate racemase, loss of the hydrogen donating ability of Glu317 by the E317Q mutation results in a $3 \times 10^4$-fold reduction in $k_{\text{cat}}/K_m$. If a similar strong stabilization, such as a LBHB is provided by the E152 residue of glutamate racemase, loss of this residue should have a significant effect on the $k_{\text{cat}}/K_m$ value of the E152Q mutant enzyme since the large difference in $pK_a$'s between the glutamine side chain and the enol intermediate would preclude the formation of a LBHB in the mutant.
Table 5.3 $V_{\text{max}}$ and $V_{\text{max}}/K_{\text{m}}$ Isotope Effects for Wild Type and Mutant Enzymes

<table>
<thead>
<tr>
<th></th>
<th>Reaction Direction</th>
<th>$V_{\text{max}}$ Isotope Effect</th>
<th>Reaction Direction</th>
<th>$V_{\text{max}}/K_{\text{m}}$ Isotope Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D-Glu $\rightarrow$ L-Glu</td>
<td>L-Glu $\rightarrow$ D-Glu</td>
<td>Ratio D$\rightarrow$L/L$\rightarrow$D</td>
</tr>
<tr>
<td>Wild Type$^a$</td>
<td></td>
<td>3.1 $\pm$ 0.5</td>
<td>2.2 $\pm$ 0.4</td>
<td>1.4 $\pm$ 0.3</td>
</tr>
<tr>
<td>D10N</td>
<td></td>
<td>3.7 $\pm$ 0.3$^b$</td>
<td>1.68 $\pm$ 0.02</td>
<td>2.2 $\pm$ 0.2$^c$</td>
</tr>
<tr>
<td>E152Q</td>
<td></td>
<td>4.2 $\pm$ 0.3</td>
<td>2.44 $\pm$ 0.07</td>
<td>1.7 $\pm$ 0.1</td>
</tr>
<tr>
<td>H186N</td>
<td></td>
<td>2.9 $\pm$ 0.2</td>
<td>4.2 $\pm$ 0.1</td>
<td>0.70 $\pm$ 0.05</td>
</tr>
</tbody>
</table>

$^a$ Data from Gallo and Knowles (1993a). $^b$Errors are standard errors of the mean ($n = 3$). $^c$Errors are standard errors of the ratio.
enzyme. The very modest 32- to 43-fold decrease in the $k_{cat}/K_m$ values of the E152Q mutant in the two reaction directions suggest this residue is not important for catalysis and a stabilizing interaction between E152 and the carboxylate oxygen of the enolate intermediate is unlikely. The most significant effect of the E152Q mutant is reflected in the order of magnitude increase in the $K_m$ value. It therefore appears that the E152 residue is important in binding the glutamate enantiomers in the active site. The fact that the isotope effects in the two reaction directions are equally affected by the mutation suggests this residue interacts with both enantiomers equally, possibly with the main-chain carboxylate of glutamate.

5.2.5 $V_{\text{max}}$ Isotope Effects

Due to the difficulties encountered with measuring the $V_{\text{max}}/K_m$ isotope effects, the $V_{\text{max}}$ isotope effects were measured for the mutant enzymes. This method compares the initial velocities of two glutamate racemase catalyzed reactions performed under the same conditions with the exception that one contains an enantiomer of [2-$^1$H]glutamate and the other contains the same enantiomer of [2-$^2$H]glutamate. The ratio between the two rates provides the $V_{\text{max}}$ isotope effect. The $V_{\text{max}}$ isotope effects are measured at high concentrations of substrate such that the value reflects all steps following substrate binding including chemistry and product release. This differs from the $V_{\text{max}}/K_m$ isotope effects which is dependent on all steps up to the first irreversible step reflecting substrate binding, chemistry and product release.

The inherent errors in the $V_{\text{max}}$ isotope effects are greater than in the $V_{\text{max}}/K_m$ isotope effects since there is a contribution from the error in enzyme concentration. The $V_{\text{max}}$ isotope effects for D10N, E152Q and H186N were measured at pH 8 in both the D $\rightarrow$ L and L $\rightarrow$ D reaction directions under initial velocity conditions using the circular dichroism assay described.
in Chapter Three. The $V_{\text{max}}$ isotope effects for E152Q are nearly equivalent to those measured for the $V_{\text{max}}/K_m$ isotope effect suggesting that an additional slow step, such as enzyme interconversion, is not rate limiting at pH 8 (Table 5.3). The ratio between the $V_{\text{max}}$ isotope effects of E152Q in the two reaction directions (1.7 ± 0.1) is the same within error as the isotope effect ratio for wild type enzyme (1.4 ± 0.3), and is consistent with the reaction profile in Figure 5.7 (b).

Interesting results are observed with the D10N and H186N mutants. Each of these mutations significantly reduce the $k_{\text{cat}}/K_m$ values, suggesting an important catalytic role in the reaction mechanism. Most significant, however, is the effect that these mutations have on the ratio between the $V_{\text{max}}$ isotope effects in the D $\rightarrow$ L and L $\rightarrow$ D reaction direction. For the D10N mutant, the isotope effect relative to the value measured for the wild type enzyme is significantly smaller in the L $\rightarrow$ D direction with a ratio between the isotope effects in the two reaction directions of 2.2 ± 0.2. This value is statistically larger than the ratio between the wild type $V_{\text{max}}$
isotope effects of $1.4 \pm 0.3$, and suggests an asymmetry has been introduced in the reaction profile describing the racemization of the enantiomers of glutamate by the D10N mutation (Figure 5.7 (c)). The diagram suggests the reaction is more cleanly rate determining in the $D \rightarrow L$ direction, implying D10 has a role in the deprotonation of D-glutamate. From earlier experiments, it is known that D-glutamate is deprotonated by C73, thus D10 is possibly involved in stabilizing the thiolate of C73.

The opposite trend is obtained from the results with the H186N mutant. The isotope effect in the $L \rightarrow D$ direction is significantly larger than that observed for wild type enzyme suggesting deprotonation is more cleanly rate determining in this direction (Figure 5.7 (d)). The ratio between the $V_{\text{max}}$ isotope effects in the $D \rightarrow L$ and $L \rightarrow D$ reaction direction has decreased from the wild type value of $1.4 \pm 0.3$ to $0.70 \pm 0.05$. H186 possibly plays a role in the deprotonation of L-glutamate which is catalyzed by C184. H186 therefore appears to stabilize the thiolate form of C184 to permit efficient abstraction of the $\alpha$-proton of L-glutamate.

The greater inherent error in the $V_{\text{max}}$ isotope effects compared to the error in $V_{\text{max}}/K_m$ isotope effects makes it difficult to conclusively establish the roles of the residues in the enzyme mechanism. For instance, the isotope effect for H186N in the $D \rightarrow L$ reaction direction is not significantly smaller than the wild type isotope effect ($2.9 \pm 0.2$ vs. $3.1 \pm 0.5$) as would be predicted by the reaction profile in Figure 5.7 (d). Conversely, the isotope effect for D10N in the $D \rightarrow L$ reaction direction is not significantly larger than the isotope effect for wild type enzyme ($3.7 \pm 0.3$ vs. $3.1 \pm 0.5$). The ratios between the isotope effects in the two reaction directions for D10N and H186N, however, are statistically different from the ratio measured for wild type enzyme. This suggests that the respective reaction profiles for D10N and H186N may be described by the diagrams in Figure 5.7 where the asymmetry is presumably induced by the loss of the stabilizing influence on the cysteine thiolates.
In summary, these results suggest D10 assists C73 in the deprotonation of D-glutamate by modulating the pK\textsubscript{a} of the cysteine residue to permit deprotonation by the thiolate form. Similarly, H186 appears to play a similar role in assisting C184 in the deprotonation of L-glutamate. Both E152 and D36 are likely involved in binding the substrate since mutation of either of these residues has little effect on catalysis but a more significant effect on binding. This was further demonstrated by the increase in the isotope effects in either reaction direction for the E152Q mutant.

5.2.6 X-ray Crystal Structure of Glutamate Racemase

An X-ray crystal structure of glutamate racemase from *Aquifex pyrophilus* with D-glutamine bound in the active site has recently been published (Hwang et al., 1999). The enzyme crystallizes as the dimer and has a single active site per monomeric unit with a loop from the other monomer inserted into each active site (Figure 5.8). A sequence alignment between the *L. fermenti* and the *A. pyrophilus* murL amino acid sequences indicates that C73, C184, D10, D36, E152 and H186 of the *L. fermenti* enzyme are conserved in the *A. pyrophilus* enzyme and correspond to C70, C178, D7, D33, E147 and H180, respectively. All residues are found within the enzyme active site with the exception of the D33 residue. The conserved E147 residue is located on the loop from the other monomer.

The glutamine inhibitor binds in a deep pocket formed by the conserved residues from the two monomers. Whereas the \(\alpha\)-proton of the natural substrate, glutamate, must be proximal to the two cysteine residues to permit deprotonation, the orientation of the D-glutamine inhibitor is reversed such that the amide group of glutamine is proximal to the cysteine residue. It is
possible that the incorrect orientation of the inhibitor affects the structure of the enzyme at the active site. Overall, no strong interactions are observed between D-Gln and the surrounding amino acid residues, accounting for the weak inhibition by this inhibitor ($K_i = 50$ mM). The authors suggest that binding of D- or L-glutamate may induce conformational changes in the active site region, leading to tighter interactions which are blocked by the crystal packing.

The residues identified by multiple sequence alignments as being highly conserved and presumably mechanistically important, D7, E147 and H180 of *A. pyrophilus* glutamate racemase, are all found within the active site of the enzyme (Figure 5.9). This is in agreement with the kinetic data which demonstrates that mutation of either D10 or H186 of *L. fermenti* significantly affects the ability of the enzyme to catalyze the efficient turnover of the enantiomers of glutamate while mutation of the E152 residue of *L. fermenti* appears to affect the binding of the substrate.
The D36 residue of \textit{L. fermenti} is presumably not in the active site, in agreement with the kinetic data which demonstrates that the D36N mutation has very little effect on the $k_{\text{cat}}$ value which is decreased by only 3-fold. The large increase in the $K_m$ value implicates a role for this residue in binding the substrate.

The D7 residue is appropriately positioned to assist the deprotonation of glutamate by C70. The sulfur atom of C70 is 4 Å away from the Oδ1 atom of D7 which, in turn, interacts with the hydroxyl group of Ser8 (3.7 Å). This corroborates our assignment which suggests the two equivalent residues of \textit{L. fermenti}, C73 and D10, are proximal to each other and may participate in a stabilizing hydrogen bond interaction between the carboxylic acid of D10 and the cysteine thiolate. We did not study the importance of Ser11, the \textit{L. fermenti} residue which corresponds to Ser8 of \textit{A. pyrophilus}.

The sulfur atom of the C178 residue interacts directly with the Oε1 atom of E147 in the other monomer, which in turn is stabilized by the Nε2 atom of H180 in the same monomer. This
conflicts with our assignments based on the experiments performed on H186N which predict C184 (C178) interacts with H186 (H180) directly. With the natural substrate bound in the active site, however, it is entirely possible that H180 and C178 interact and that the E147 residue which is located on a loop from the other monomer occupies a different location. The authors indicate that a local flexibility is apparent in this region which may influence the relative positions of the residues in the enzyme active site particularly in the presence of an incorrectly bound inhibitor.

A direct interaction between C178 and E147 which is important for catalysis, as suggested by the authors, seems unusual in light of the observation that mutation of E152, the residue equivalent to E147 of the \emph{A. pyrophilus} enzyme, has very little effect on the specificity constant (Table 5.2). Such a result would not be expected if a direct interaction between C184 and E152 is catalytically important. Since the E147 residue is located on a loop, the position of the loop may be sufficiently displaced by the bound inhibitor. The authors note that when D-Glu is modelled into the crystal structure, the main-chain carboxyl group of the substrate is accessible to the Oe1 atom of E147. An interaction between E152 and bound glutamate in the enzyme active site may indeed be possible as suggested by the studies with the E152Q mutant.

Although direct structural evidence is not available to corroborate all the assignments described in this chapter, the presence of the residues in the active site and their proximity to the respective cysteine residues provides a good indication that these residues are important in the mechanism catalyzed by the enzyme.
5.3 Active Site Residues in Amino Acid Racemases

5.3.1 DAP Epimerase

The crystal structure of DAP epimerase from *Haemophilus influenzae* has recently been determined (Cirilli *et. al.*, 1998). The enzyme crystallizes as a monomer in an oxidized form with a disulfide bridge between two cysteine residues, C73 and C217. These residues are in their reduced form in the active enzyme and serve as the general acid/base catalysts involved in the deprotonation/reprotonation of the DAP epimers. Two residues, H159 and E208, are found in the active site on opposite sides of the plane of the disulfide bond. The authors suggest that each of these two residues may electrostatically interact with the \( \alpha \)-carboxyl and \( \alpha \)-amino groups of DAP. Alternatively, these residues may modulate the \( pK_a \) values of the two catalytic cysteine residues in the reduced enzyme. No studies have yet been published which examine the roles of these residues in the epimerization reaction catalyzed by this enzyme.

5.3.2 Alanine Racemase

Very recent experimental evidence by Toney suggests hydrogen bonding interactions are important in the stabilization of the phenolate anion of Tyr265 of alanine racemase (Sun and Toney, 1999), which, together with Lys39, acts as the general acid/base catalyst of the PLP-dependent *B. stearothermophilus* alanine racemase. The X-ray crystal structure displays an interaction between Tyr265 and Arg219 mediated by His166 through hydrogen bonds (Shaw *et. al.*, 1997). This hydrogen bonding network is presumed to lower the \( pK_a \) of tyrosine to \(~7\) by electrostatic stabilization of the phenolate anion. Mutation of the arginine residue disrupts the
Table 5.4 Primary Isotope Effects for Wild Type and R219 Mutants of Alanine Racemase

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<td>(V_{\text{max}}/K_m) Isotope Effect</td>
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<td>Wild Type</td>
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<td>1.3 ± 0.1</td>
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<td>1.8 ± 0.1</td>
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<tr>
<td>R219E</td>
<td>1.1 ± 0.02</td>
<td>1.4 ± 0.07</td>
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</table>

\(^a\)Data from Sun and Toney (1999).

hydrogen bond network, influencing the ability of Tyr265 to act as a general base in the L → D direction, and demonstrates the importance of the hydrogen bonding network for the effectiveness of Tyr265 as a general base.

The isotope effects for the three arginine mutants, R219K, R219A and R219E, were determined in both reaction directions (Table 5.4). In the D → L direction, the isotope effects are unchanged relative to those observed for the wild type enzyme, whereas the isotope effects measured for the mutants in the L → D direction, in which Tyr265 acts as the general base, are significantly larger than wild type. This suggests that abstraction of the \(\alpha\)-proton in the L → D direction is at least partially rate limiting for the mutant enzymes (and may be described by the reaction profile in Figure 5.7 (d)). Furthermore, the pH profiles for each mutant show an increase in the \(pK_a\) of an ionizable group, presumably the phenolic Tyr265 hydroxyl, from ~7 for the wild type and R219K mutant enzymes to ~10 for the R219A and R219E mutants. Disruption of the hydrogen bonding network by an arginine mutation affects the \(pK_a\) of the tyrosine hydroxyl group which increases to the solution value for tyrosine (\(pK_a \approx 10\)). Similar results may
be observed with the D10N and H186N mutants of glutamate racemase which are proposed to have a stabilizing influence on the cysteine thiolates.

5.4 Further Studies

The roles assigned to certain residues in the active site of glutamate racemase are dependent on measurements on $V_{\text{max}}$ isotope effects, however, the large inherent error in these measurements makes it difficult to draw any firm conclusions. The role of the D10 and H186 residues, in particular, require further examination. It would be useful to determine the $V_{\text{max}}/K_m$ isotope effects for both D10N and H186N since the asymmetry may be more fully expressed on this parameter. This may be particularly true if an additional slow step, such as enzyme interconversion, masks the isotope effect on $V_{\text{max}}$. Although technical difficulties do not allow the $V_{\text{max}}/K_m$ isotope effects to be determined using the competitive deuterium washout experiment, the initial velocity of L- or D-glutamate at various substrate concentrations with either unlabelled or deuterated glutamate may be used to determine the isotope effect on $V_{\text{max}}/K_m$ (Cleland, 1979). It may then be possible to more firmly establish the participation of either of these residues in the deprotonation of the enantiomers of glutamate by their interaction with the respective cysteine thiolate residues.

It would be interesting to examine the isotope effects for the D36N mutant of glutamate racemase. The large $K_m$ for this mutant implicates an important role for this residue in binding the substrate and mutation may result in an increase in the observed isotope effects in the two reaction directions, much like that observed for the E152Q mutant. Further studies may await the release of the X-ray crystal structure coordinates to determine the precise location of this residue.
Finally, it may be useful to investigate the importance of other conserved residues of *L. fermenti* glutamate racemase. It would be particularly interesting to investigate the importance of residues which may participate in a stabilizing hydrogen bonding interaction, potentially of the low barrier type, with the enolate intermediate. The X-ray crystal structure of *A. pyrophilus* glutamate racemase indicates that the strictly conserved residue Tyr39, which corresponds to Tyr42 of *L. fermenti* glutamate racemase, is located in the active site. Although the authors do not suggest a role for this residue, it is conceivable that a tyrosine residue, with a solution $pK_a$ of $\sim 10$, could be a hydrogen donor. Matched $pK_a$'s between the enol tautomer of glutamate and the tyrosine side chain could provide significant stabilization by the formation of a LBHB between tyrosine and the enolate oxygen. An appropriate mutant which disrupts the hydrogen donating character at this position should result in significantly impaired racemization and an increase in the observed isotope effects in the two reaction directions.

### 5.5 Conclusion

The experiments described in this thesis demonstrate an effective means by which the asymmetry induced in a reaction profile by site-directed mutagenesis of appropriate residues may be used to determine the roles of active site residues in the reaction catalyzed by glutamate racemase. From the results of experiments which utilized either alternate substrates or isotopically labelled substrates, we have determined that Cys73 in *L. fermenti* glutamate racemase is responsible for the deprotonation of D-glutamate and that the thiolate form of this residue may be stabilized by an interaction with the adjacent Asp10 residue. Conversely, Cys184 is responsible for the deprotonation of L-glutamate and this residue may have a stabilizing interaction with His186.
5.6 Experimental Methods

5.6.1 Sequence Alignments

Sequence alignments were performed online using the CLUSTAL W 1.74 algorithm courtesy of The Infectious Diseases Division, Washington University, St. Louis, MO (http://www.ibc.wustl.edu/service/msa/index.html).

5.6.2 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:

09 GGAGTGATGAATTCTGGCTTGG
10 CCAAGCCAGAATTCATCACTCC
11 CTTCGTGGGCAACCAAGGTAC
12 GTGACCTTGGTTGCCCACGAAG
13 CGAAATCGTTCAGCACGGCC
14 GGCCGTGCTGAACGATTTCG
15 GGGCTGCACCAACTTCCCG

5.6.3 Site-Directed Mutagenesis

Mutants were prepared by the recombinant circle PCR technique of Jones and Winistorfer (1992). The pKG3 vector bearing the \textit{murI} gene was linearized in two separate reactions with either HindIII (Gibco-BRL) or EcoR1 (Promega). Four PCR reactions were performed on the HindIII linearized DNA: reaction 1 (D10N) contained primer 05, described in Chapter Three, and primer 10, reaction 2 (D36N) contained primers 05 and 12, reaction 3 (E152Q) contained primers 05 and 14 and reaction 4 (H186N) contained primers 05 and 16. The EcoR1 linearized DNA was amplified using the following primer pairs: reaction 5 (D10N) contained primer 06, described in Chapter Three, and primer 09, reaction 6 (D36N) contained primers 06 and 11, reaction 7 (E152Q) contained primers 06 and 13 and reaction 8 contained primers 06 and 15. PCR reactions (100 μL total) contained 20 ng template, 10 pmol each primer, 0.4 mM of each dNTP, 2.5 μL dimethyl sulfoxide and 2.5 U Pwo DNA polymerase (Boehringer-Mannheim) in the supplied PCR buffer containing magnesium sulfate (2 mM final magnesium sulfate concentration). DNA was amplified using an M J Research MiniCycler by an initial denaturation step at 96°C for 2 minutes followed by 10 cycles of denaturation at 96°C for 1 minute, annealing at 60°C for 45 seconds and extension at 72°C for 2 minutes. Another 10 cycles of amplification were performed with a 45 second 55°C annealing step followed by
another 10 cycles of amplification with a 45 second 50°C annealing step. After the last cycle, an additional 7 minutes at 72°C ensured complete extension. Recombinant DNA plasmids were obtained by combining the products of reactions 1 and 5 (D10N), 2 and 6 (D36N), 3 and 7 (E152Q) and 4 and 8 (H186N). The combined PCR products were denatured at 94°C for 3 minutes and annealed at 50°C for 2 hours. CaCl₂-competent *E. coli* DH5α bacterial cells were transformed with the recombinant DNA plasmid. 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 broth containing 50 μg/mL ampicillin.

5.6.4 Isolation and Purification of Mutant Enzymes

The overnight bacterial cultures were screened for glutamate racemase activity by the method described in Chapter Three. Plasmid DNA was isolated from cultures displaying activity at a lower level than a wild type control sample. D10N mutants were screened by treating the plasmid DNA with EcoRI and analyzing the DNA fragments by agarose gel electrophoresis on a 1% agarose gel visualized with ethidium bromide. Plasmid DNA was sequenced using primers 07 and 08 described in Chapter Three. Four plasmids with the correct mutation, pSG03 (D10N), pSG04 (D36N), pSG05 (E152Q) and pSG06 (H186N) were obtained.

The mutant enzymes were purified by the method described in Chapter Three. The purity of the racemases was assessed by SDS-polyacrylamide gel electrophoresis on a 12% acrylamide gel using a Bio-Rad Mini-PROTEIN II electrophoresis system. Protein bands were visualized by staining with Coomassie Brilliant Blue. The molecular weight of the pure racemase was determined by electrospray ionization mass spectrometry on a PE-SCIEX API 300 MS operated by Shouming He in the laboratory of Dr. Stephen Withers, Department of Chemistry, UBC.
5.6.5 Kinetic Constants for Mutant Glutamate Racemases

The kinetic constants for each mutant enzyme were determined using the circular dichroism assay described in Chapter Three in both the D → L and L → D reaction directions. The reaction contained 0.2 mM DTT and glutamate concentrations varying from the lower sensitivity limit of 1 mM up to 5 X \( K_m \) in 10 mM potassium phosphate pH 8 buffer. The reaction was initiated with 0.06 mg D10N, 0.003 mg D36N, 4.75 x 10^{-4} mg E152Q or 0.08 mg H186N.

5.6.6 \( V_{max}/K_m \) Isotope Effects

The \( V_{max}/K_m \) isotope effect for E152Q was measured using the competitive deuterium washout experiment described in Chapter Four. The CD signal of a 250 \( \mu \)L solution equilibrated at 30°C containing 5 mM deuterated substrate and 5 mM undeuterated enantiomer in 10 mM potassium phosphate buffer, pH 8, with 0.2 mM DTT, was monitored at 210 nm following the addition of 2.4 x 10^{-3} mg of E152Q. The double competitive deuterium washout experiment monitored the CD signal at 210 nm of a 250 \( \mu \)L solution containing 5mM of each deuterated in 10 mM potassium phosphate buffer, pH 8, following the addition of 2.4 x 10^{-3} mg of E152Q.

5.6.7 \( V_{max} \) Isotope Effects

The \( V_{max} \) isotope effects were determined in both reaction directions by comparing the initial velocity of a reaction containing 20 mM of an enantiomer of [2-\(^1\)H]glutamate to one containing 20 mM of the same enantiomer of [2-\(^2\)H]glutamate. The ellipticity of a solution (250 \( \mu \)L) equilibrated to 30°C containing glutamate and 0.2 mM DTT in 10 mM potassium phosphate
buffer, pH 8, was monitored at 210 nm using a JASCO 710 Circular Dichroism Spectrophotometer. The reaction was initiated by the addition of the appropriate enzyme (0.04 mg D10N, 9.5 x 10^{-4} mg E152Q or 0.09 mg H186N). The rate was determined from the slope over the first 10% of the reaction.

5.6.8 Error Analysis

Three replicates of each experiment were performed to obtain a mean value with the error described by the standard error (SE) of the mean. The standard error in the ratio between kinetic isotope effects (KIE) in the two reaction directions was calculated by

\[
\text{SE}\left(\frac{\text{KIE}_{(D\rightarrow L)}}{\text{KIE}_{(L\rightarrow D)}}\right) = \frac{\left(\frac{\text{SE}\ KIE_{(D\rightarrow L)}}{\text{KIE}_{(L\rightarrow D)}}\right)^2}{\left(\frac{\text{SE}\ KIE_{(L\rightarrow D)}}{\text{KIE}_{(D\rightarrow L)}}\right)^2 + \left(\frac{\text{SE}\ KIE_{(L\rightarrow D)}}{\text{KIE}_{(D\rightarrow L)}}\right)^2}.
\]

Statistical difference between two means was evaluated by a Student’s t-test at a 90% confidence limit with 4 degrees of freedom.


Appendix A

Amino acid sequence of *Lactobacillus fermenti* glutamate racemase

MDNRPIGVMDSGLGGLSVVRVIQQKLPNEEVIIFVGDQGHFPYGTKDQAEVRQLALSIGAFFKHDVKKMVMVACNTATAAALPALQAALPPIPVGIEPGARAALAQDKKGPIGVIATTATTAGAYPATIERLAPGTPVIAKATQPMVIEVEHGQTGTAKAQEVVSEQLMTFKEHPVKTLMIGCThFPLAPEISKAVGPVTVALVDPAKETVATASKWLEQHQAMGNHAPNYHLYSTGNLPLRAGVNWLLSGHDILGTAAQIEEDG

Tryptic fragments of glutamate racemase are:

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<td>SWLEQHQAMGNHAPNYHLYSTGNLPLDRL</td>
</tr>
<tr>
<td>143-173</td>
<td>3387.69</td>
<td>ATQPMVIEVEHGQTGTAKAQEVVSEQALMTFK</td>
</tr>
<tr>
<td>64-101</td>
<td>3809.06</td>
<td>HDVKMMVVACNTATAAALPALQAALPIPVIGVIEPGAR</td>
</tr>
<tr>
<td>217-250</td>
<td>3855.85</td>
<td>SWLEQHQAMGNHAHPNYHLYSTGNLPDLRAGVNM</td>
</tr>
<tr>
<td>68-108</td>
<td>4027.19</td>
<td>MMVVACNTATAAALPALQAALPIPVIGVIEPGARAALAQDK</td>
</tr>
<tr>
<td>210-245</td>
<td>4086.96</td>
<td>ETVATAKSWLEQHQAMGNHAHPNYHLYSTGNLPDLR</td>
</tr>
</tbody>
</table>
Appendix B

Normal complement of *Lactobacillus fermenti murl* indicating the binding sites for each of the primers discussed in the thesis.

```
ATG GAC AAT CGC CCA ATT GGA GTG ATG GAT TCT GCC TTG GGG GCC CTT TCA GTC GTC
met asp asn arg pro ile gly val met asp ser gly leu gly leu ser val val

CGG GTT ATC CAG CAA AAA CTA CCC AAC GAA GAG GTC ATC TTC GTG GCC GAC CAA GGT
arg val ile gln gln lys leu pro asn glu glu val ile phe val gly asp gln gly

CAC TTC CGG TAC GGA ACC AAG GAT CAA GGG GAG GTC ATC TTC GTG GCC CCA TCG ATT
his phe pro tyr gly thr lys asp gln ala glu val arg gln leu ala leu ser ile

AGG GCC GCC GCA CTG CCG GCC CTG CAA GCG GCC CCC ACC ACG GCA ATC GGG GTC
thr ala ala ala leu pro ala leu gln ala ala leu pro ile pro val ile gly val

ATT GAG CCC GCC CGG GCC CTG GCC CAG GAT AAA AAG GGA CCG ATT GGG GTG
ile glu pro gly ala arg ala ala leu ala gln asp lys gly pro ile gly val
```

```
GCG CCG GCC ACC ACC CCG GCC GCC TAC GCC GCT ACG ATC GAA CGG CTA
ile ala thr thr ala thr thr thr ala gly ala tyr pro ala thr glu arg leu

GCG CCG GGT ACC CCG GCC ACC GCC CAG AAG GCC ACC CAG CCA ATG GTC GAA ACG ATC GTT GAG
ala pro gly thr pro val ile ala lys ala thr gln pro met val glu ile val glu
```

```
CAC GCC CAG ACC GGG ACG GCC AAG GCC CAA GAA GTG GTT AGT GAA CAG CTA ATG ACC
his gly gln thr gly thr ala lys ala glu val val ser glu gln leu met thr
```
TTT AAA GAG CAC CCG GTA AAA ACC CTG ATT ATG GGC TGC ACC CAC TTC CCG TTT TTG
phe lys glu his pro val lys thr leu ile met gly cys thr his phe pro phe leu

GCC CCG GAA ATA AGT AAG GCG GTC GGA CCA ACG GTG GCC GAC CCC GCT AAG
ala pro glu ile ser lys ala val gly pro thr val ala leu val asp pro ala lys

GAA ACG GTG GCC ACG GCT AAG TCC TGG CTG GAA CAG CAC CAA GCA ATG GGT ACG CAC
glu thr val ala thr ala lys ser trp leu glu gln his gln ala met gly asn his

GCC CAC CCC AAC TAC CAC CTA TAT TCA ACG GCC AAC CTC CCT GAC TTA AGG GCC GGG
ala his pro asn tyr his leu tyr ser thr gly asn leu pro asp leu arg ala gly

GTG AAT AAG TGG TTG TTG TCG GGG CAC TTT GAC TTA GGA ACC GCC CAA ATT GAA GAG
val asn lys trp leu leu ser gly his phe asp leu gly thr ala gln ile glu glu

GGG GAT TAA
gly asp OCH
Appendix C

pUC18 Sequence  length 2686 bases

---

1 GCGCCCAATA CGCAAACCGC CTCTCCCCGC GCGTTGGCCG ATTCATTAAT GCAGCTGGCA
61 CGACRGGTTT CCGGACTGGA AAGCGGCGAC TGAGCAGCACC GCRATTAAAG TGGGTTAGCT
121 CACCTTATTG GCACCAGGCGG CTTTACACCT TATGCTTCCG GCTCGTATGT TGTTGGAAT
181 TCGAGCCCGA AACACATTTAC ACAGACAGAAA CAGAGTATGAC CATGATTACG AATTCGAGCT

BamHI -insertion site for muri

241 CGGTACCGGG GGATCCTCTA GAGTCGACCT GCAGGACATGA AAGCTTGGCA CTGGCCGTCG
301 TTACCACGCAC CGTGACTGGC AAGACGCCCTG GCGTTACGCC ACTTAATCGC CTTGCAGCAC
361 ATCCCCCTTT CGCCAGCTGG CACAAATAGCG CACGGCGCCT CAGCACCGCT GTGCTGGTCC
421 CGGCACTCCCG TTTCAGAAGAC TCTGTGAGCG TGACGACGAG CAGCTATGAG ACATGATTACG
481 CGGTATTTCT ACACCCCTGG TAGTCGACCT TCAGTGACAT CGACTCAGAC TCGGGGCATT
541 TGAGGACAGCG CCGACACCCG CCAACACCCG CTGACGCGCC CTGACGGGCT TGTCTGCTCC
601 CGGCATCCCCG TTTCAAGGACG GCAGGCCCCT GCTCCGGGAC CTGCATGTGT CAGAGGTTTT
661 CACCCTCGTC ACCGAAACCG CGGAGACGAA AAGGCGCTGT GATAAGCGCT TTTTATAGG
721 SSAATGCTCAT GTATAAAATTT TTTCTTCAGA CTGTCAGGCG CAATTTTGGC GAAATAGGC
781 GCGGAACCCC TATTTGTGCA TTTTCTTTAAA TACATTCAATA TATGTATCCT CGACGAGAC
841 AATAACCCCTG ATAAATGCTT CAAATAATGG AAAAACGGAA GAGTATGAGT ATTCAACATT
901 TCGGTGTCGCG CCTATTCGCC TTTTTGGCCG CATTGCTTCT CCGGACTGCG ACGCACCGAC
961 AAAGCGCTGGT GAAAGTAAAAG GATGCTAGTCA ATCAGTTGGG TGCGAGAGTG GCTGATGAC
1021 AACTGGATCT CAACAGCGCGT AAGATCTTCTG AGAGTTCCTCG CCCGAAGAAG CGTGGTCCAA
1081 TGATGAGCCAC TTTTAAAGTT GTGTGCTATTG GCCGGTATGG ATCCCGTAGT GACGCGGCGC
1141 AAGAGCAACT CGGTGGCCGGC ATACACTATT CTCAATGATG CTTGGTTGAG TACTCACAG
1201 TCACAGAAAA GCATCTTACG GATGCGATCG ATGTAAGTGA AAGTGGATG AGATGGTGGC
1261 CCATGACTGC TAACACTGCG GCCAACCTTAC TCTGACACCA GATCGGAGGA CCGAGAGGAC
1321 TAACCGCTTTTT TTTCAAGACC ATGGGAGGATC ATGTAAGCTG CCGGACTGCG TGGGAAGCGG
1381 AGCTGAATGA AGCCATACCA AACGACGAAGC GTGACACCAC GATGCTGTTA GCAATGCGAA
1441 CAAAGTTGCG CAAACTATTACA CTGGGCGAAC TACTTACTCCT AGCTGCTCCGG CAACAATTAA
1621 CACTGGGCG ACAATGCTTAA CTTCCCGCTA ATCTGCTTCCCT CACCAAGTGG
1681 CAACATAGGA TGAACGGCAAT AGACAGATCG CTGAGTCAAG TGCCCTCAGCT ATTAAACATT
1741 GGTAACTGTC AGACCAAGTT TACTCATATA TACTTTAGAT TGATTTAAAA CTTTTTTTTT
1801 AATTTAAAAA GATCTAGGTG AAGATCTTTT TTTGATAATCT CATGACCAAA ATCCCTTAAC
1861 GTGAGTTTTT GTCAGCTGGA GCGTGAGACC CCGTAGAAAA GATCAAAGGA TCTTCTTGGAG
1921 ATCCCTTTTT TCTGCGCTGA ATCTGCTGGT TGCAAACAAA AAAACCACCG CTCACCGCGG
1981 TGGTTTTTGG GCGGATCAA GAGCTACCAA CTCTTTTTCC GAAGGTAACGT GGTTCAGCA
2041 GAGCGCAGAT ACCAAATACGT GTCCTTCTAG TGTAGCCGTA GTTAGGCCAC CACTTCAAGA
-----------------06-----------------
2101 ACTCTGTGAC ACCCGCTACA TACCTCGTCT TGCTAAATCT GTTACCAGTG GCTGCTGCCA
2161 GTGCCTGATAA GTGCGTTTTT ACCGGGTGGG ACTCAAGACG ATAGTTACCG GATAAGGCGG
2221 AGCGGCTCGG CTGAACGCGG GGGTGGTCGC AAGAGCCCAG CTTGAGGCGA AGCAGCTACA
2281 CCGAACTGAG ATACCTACAG CGTGAGCTAT GAGAAGGCC CACGCTTTCC GAAAGGAGAA
2341 AGGCCGACAG GTATCCCGTA AGCCGCAGGG TCGGAACAGG AGAGCGCCAG AGGGAGCTTC
2401 CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTGCGTTT TCGCCACCTC TGACTTGAGC
2461 GTGATTTTTT GTGATGCTCG TCGAGGGGGG GAGCCCTATG GAAAAACCAC AGCAACGCGG
2521 CTTTTTACGG TGTCTGCGCC TTTTGCTGCC CTTTTGCTCA CAGTGTCTTT CTCGCGTTAT
2581 CCCCCTGATTG TGTTGATAAC CGTATTACCG CTTTTGAGTG AGCTGATACC GCTCGCCGCA
2641 GGGCCACGAC CGAGCGACGC GAGTCACTGA GCCAGGAAGC GGAAGA
Appendix D

Theory

For an enzymatic reaction consisting of the following sequence of interconversions where the interconversion of the two enzyme forms is assumed to be rapid such that they may be considered as the same species:

\[
E_1 \xrightarrow{k_1} E_2 \xrightarrow{k_2} E_3 \xrightarrow{k_3} E_1
\]

(Scheme A.1)

A "net rate constant", \( k' \), can be described which is essentially a rate constant which would produce the same flux through that step if the step were irreversible. Therefore;

\[
E_1 \xrightarrow{k'} E_2 \xrightarrow{k'_2} E_3 \xrightarrow{k'_3} E_1
\]

(Scheme A.2)

The fraction of enzyme in any form can be computed by:

\[
\frac{[E_1]}{[E]} = \frac{1/k'_1}{1/k'_1 + 1/k'_2 + 1/k'_3}
\]

(Equation A.1)

since \( v = E_n k'_n \)

\[
v = \frac{[E_3]}{1/k'_1 + 1/k'_2 + 1/k'_3}
\]

(Equation A.2)

Calculation of Net Rate Constants

The net rate constant, \( k' \), is the true rate constant, \( k \), multiplied by the portion of the enzyme form that partitions forward as opposed to the reverse reaction. Therefore, the net rate constant, \( k'_1 \), for the conversion of \( E_1 \) to \( E_2 \) in the following enzymatic reaction

\[
E_1 \xrightarrow{k_1} E_2 \xrightarrow{k_2} E_3 \xrightarrow{k_3} E_1
\]

(Scheme A.3)

is:

\[
k'_1 = k_1 \left( \frac{k'_2}{k'_2 + k_1} \right)
\]

(Equation A.3)

The reaction catalyzed by glutamate racemase may be described by,

---

1 The derivation of the net rate constants is taken from Cleland, W.W. (1975) *Biochemistry* 14, 3220-3224.
\[ \begin{align*}
E_1 & \xrightarrow{k_1[S]} E_1S & \xrightarrow{k_2} E_2P & \xrightarrow{k_3} E_2 + P
\end{align*} \]
(Scheme A.4)

where the net rate constants are:
\[
k_3, \quad \frac{k_2}{k_2 + k_3}, \quad \frac{k_1k_2k_3[S]}{k_1(k_2 + k_3) + k_2k_3}
\]

**Calculation of \( V_{\text{max}} \)**

From equation (A.2),
\[
V_{\text{max}} = \frac{[E_i]}{k_3 + \left( \frac{k_2k_3}{k_2 + k_3} + \frac{k_1k_2k_3[S]}{k_1(k_2 + k_3) + k_2k_3} \right)}
\]
(Equation A.4)

\( V_{\text{max}} \) measured at \([S] \to \infty\), therefore
\[
V_{\text{max}} = \frac{[E_i]k_2k_3}{k_2 + k_2 + k_3}
\]
(Equation A.5)

If \( k_3 \) is very large compared with \( k_2 \) and \( k_2 \) (if substrate dissociation is much faster than the chemical steps of the reaction), the \( V_{\text{max}} \) isotope effect reduces to the intrinsic isotope effect, \( ^{1}H_2/k_2^{3}H_2k_2 \).

**Calculation of \( V_{\text{max}}/K_m \)**

\( V_{\text{max}}/K_m \) is the apparent first-order rate constant for the reaction of enzyme and substrate when the substrate is at a very low concentration. At low concentrations of substrate, all the enzyme will be in the \( E_1 \) form.

\[
\nu = \frac{V_{\text{max}}}{K_m + [S]}
\]
(Equation A.6)

which reduces to \( \nu = \frac{V_{\text{max}}}{K_m} [S] \) at low substrate concentrations.

\[
\frac{V_{\text{max}}}{K_m} = \frac{\nu}{[S]} \quad \text{and} \quad \nu = k_1[E_i]
\]

\[
k_1 = \frac{k_1k_2k_3[S]}{k_1(k_2 + k_3) + k_2k_3}
\]
(Equation A.7)

\[
\frac{V_{\text{max}}}{K_m} = \frac{k_1k_2k_3[E_i]}{k_1k_2 + k_1k_3 + k_2k_3}
\]
(Equation A.8)
where \( \frac{k_1k_2k_3}{k_1k_2 + k_1k_3 + k_2k_3} = k_{1,2,3} \) (Equation A.9)

The composite rate constant, \( k_{1,2,3} \) describes the reaction rate over the whole series of transition states in the reaction and if written as the sum of the reciprocal rate and equilibrium constants, each term describes the free energy difference between the \( n \)th transition state and the original reactant and is defined as:

\[
\frac{1}{k_{1,2,3}} = \frac{1}{k_1} + \frac{1}{k_2k_1} + \frac{1}{k_3k_{1,2}} \quad \text{where} \quad K_1 = \frac{k_1}{k_{-1}} \quad \text{and} \quad K_{1,2} = \frac{k_1k_2}{k_{1,2}}
\]

Using composite rate equations, the \( V_{\text{max}}/K_m \) isotope effect is described by \( ^{1}Hk_{1,2,3}/^{2}Dk_{1,2,3} \).