MECHANISTIC ASPECTS OF CARBOHYDRATE EPIMERIZATION

by

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We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

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Department of CHEMISTRY

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Date Nov. 22/2002
Abstract

Epimerases are enzymes that invert the stereochemistry at a stereogenic center in a molecule with two or more chiral centers. Most known sugar epimerases catalyze this reaction by simple deprotonation/reprotonation or by oxidation/reduction mechanism. Only two carbohydrate epimerases are known that catalyze the inversion of stereochemistry by mechanisms different from those mentioned above. These are: L-ribulose-5-phosphate 4-epimerase and UDP-N-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase).

L-Ribulose-5-phosphate 4-epimerase (L-Ru5P 4-epimerase) is a bacterial enzyme that interconverts L-ribulose 5-phosphate (L-Ru5P) and D-xylulose 5-phosphate (D-Xu5P) by a retroaldol/aldol mechanism. The epimerase shares 26% sequence homology and a high degree of structural homology with the metal-dependent class-II aldolase, L-fuculose-1-phosphate aldolase, which catalyzes the reversible C-C bond cleavage of L-fuculose 1-phosphate (L-Fu1P) to give L-lactaldehyde and dihydroxyacetone phosphate. We have shown that the epimerase and the aldolase share a conserved phosphate binding pocket. Since the substrates of the epimerase and the aldolase are phosphorylated at opposite ends, the epimerase binds the substrate in a reverse orientation as compared to the aldolase. Due to this “flipped” orientation of the bound substrates, the two enzymes utilize different acid/base residues for catalyzing the reaction. Asp120' has been identified as the catalytic residue responsible for deprotonating D-Xu5P. Thus, while there is a single catalytic residue, Glu73, in the aldolase, there are two different catalytic residues, Asp120' and Tyr229' (Cleland et al.) in the epimerase.

UDP-N-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase or UDPE) is a homodimeric, bacterial enzyme that catalyzes the interconversion of UDP-N-
acetylglucosamine (UDP-GlcNAc) and UDP-N-acetylmannosamine (UDP-ManNAc) by a mechanism involving C-O bond cleavage/formation. UDPE is allosterically regulated by its own substrate UDP-GlcNAc. Using site-directed mutagenesis we have identified two residues, His213 and Lys15, which play an important role in substrate binding. Three carboxylate mutants, D95N, E117Q and E131Q, showed a 10,000-fold decrease in catalytic efficiency and an impaired allosteric control. The ability of the E117Q mutant to catalyze the release of intermediates from UDP-GlcNAc at rates comparable to that of the wild-type enzyme suggests that Glu117 could be the catalytic residue responsible for deprotonating UDP-ManNAc. The wild-type epimerase shows a 50% increase in fluorescence with 1 mM of a 10:1 mixture of UDP-GlcNAc and UDP-ManNAc. On the basis of kinetic and binding studies with the wild-type epimerase and the three carboxylate mutants we have shown that the enzyme is capable of binding UDP-ManNAc in the absence of UDP-GlcNAc. We propose that UDPE is a V-system in which binding of UDP-GlcNAc to one subunit increases the catalytic efficiency of the other subunit.
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## Abbreviations and Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>Å</td>
<td>Angstrom(s)</td>
</tr>
<tr>
<td>δ</td>
<td>Chemical shift (in nmr)</td>
</tr>
<tr>
<td>ε</td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine 5'-monophosphate</td>
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<tr>
<td>araA</td>
<td>Gene encoding L-arabinose isomerase</td>
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<tr>
<td>araB</td>
<td>Gene encoding L-ribulokinase</td>
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<tr>
<td>AraB</td>
<td>L-ribulokinase</td>
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<tr>
<td>araBAD</td>
<td>The group of araA, araB and araD genes</td>
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<td>araC</td>
<td>Gene encoding the regulatory protein for the araBAD operon</td>
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<td>araD</td>
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<td>Asp120 residue in L-Ru5P 4-epimerase, coming from the subunit adjacent to the one bearing the active-site Zn^{2+}</td>
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<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BGT</td>
<td>β-Glucosyltransferase</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair (in DNA)</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CD</td>
<td>Circular dichroism</td>
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<tr>
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<td>Column volume(s)</td>
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<tr>
<td>d</td>
<td>Doublet (in nmr)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>D76E</td>
<td>L-Ru5P 4-epimerase in which an aspartate at position 76 has been replaced by a glutamate</td>
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<td>Da</td>
<td>Dalton(s)</td>
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<td>Desorption chemical ionization mass spectrometry</td>
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<td>DHA</td>
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<td>E142Q</td>
<td>L-Ru5P 4-epimerase in which a glutamate at position 142 has been replaced by a glutamine</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid, disodium salt</td>
</tr>
<tr>
<td>Enz</td>
<td>Enzyme</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron Paramagnetic Resonance</td>
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<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
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<tr>
<td>GAP</td>
<td>Glyceraldehyde 3-phosphate</td>
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<tr>
<td>Gene/Enzyme</td>
<td>Description</td>
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<td>α-GDH</td>
<td>α-glycerophosphate dehydrogenase</td>
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<td>GDP</td>
<td>Guanosine 5′-diphosphate</td>
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<td>GlcNAc</td>
<td>N-acetylg glucosamine</td>
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<td>H95N</td>
<td>L-Ru5P 4-epimerase in which a histidine at position 95 has been replaced by an asparagine</td>
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<td>L-Ru5P 4-epimerase in which a histidine at position 97 has been replaced by an asparagine</td>
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<td>UDP-GlcNAc 2-epimerase in which a histidine at position 213 has been replaced by an asparagine</td>
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<tr>
<td>His218'</td>
<td>His218 residue in L-Ru5P 4-epimerase, coming from the subunit adjacent to the one bearing the active-site Zn^{2+}</td>
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<td>H218N</td>
<td>L-Ru5P 4-epimerase in which a histidine at position 218 has been replaced by an asparagine</td>
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<td>hr(s)</td>
<td>Hour(s)</td>
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<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N′-[2-ethane sulphonic acid]</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure/performance liquid chromatography</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl-1-thio-β-galactopyranoside</td>
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<tr>
<td>K15A</td>
<td>UDP-GlcNAc 2-epimerase in which a lysine at position 15 has been replaced by an alanine</td>
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<td>K42M</td>
<td>L-Ru5P 4-epimerase in which a lysine at position 42 has been replaced by a methionine</td>
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<tr>
<td>$k_{cat}$</td>
<td>Catalytic rate constant (turnover number)</td>
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<tr>
<td>kDa</td>
<td>Kilodalton(s)</td>
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<tr>
<td>$K_i$</td>
<td>Dissociation constant for an enzyme-inhibitor complex</td>
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<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
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<tr>
<td>$K_m,_{app}$</td>
<td>Apparent value of Michaelis constant</td>
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$K_s$ Dissociation constant for enzyme-substrate complex
LB Luria Bertani medium
L-Ru5P L-ribulose 5-phosphate
LPS Lipopolysaccharide
LSIMS Liquid secondary ion mass spectrometry
m Multiplet (in nmr)
ManNAc N-acetylmannosamine
min Minute(s)
mRNA Messenger ribonucleic acid
MWCO Molecular weight cut-off
N28A L-Ru5P 4-epimerase in which an asparagine at position 28 has been replaced by an alanine
NAD$^+$ Nicotinamide adenine dinucleotide
NADH Nicotinamide adenine dinucleotide, reduced form
NADP$^+$ Nicotinamide adenine dinucleotide phosphate
NADPH Nicotinamide adenine dinucleotide phosphate, reduced form
NAPS Nucleic Acid and Protein Services
nfrC Gene encoding UDP-GlcNAc 2-epimerase
NMR Nuclear magnetic resonance
OD$_{600}$ Optical density measure at 600 nm
PAGE Polyacrylamide gel electrophoresis
PCR Polymerase chain reaction
PLP Pyridoxal phosphate
psi Pounds per square inch
QSDM QuickChange™ Site-directed Mutagenesis
R relaxed (state or form of enzyme)
RCPCR Recombinant circle polymerase chain reaction
rffD Gene encoding UDP-ManNAc dehydrogenase
rffE Gene encoding UDP-GlcNAc 2-epimerase
RMSD Root mean square deviation
RNA Ribonucleic acid
RPEase D-Ribulose-5-phosphate 3-epimerase
rpm Rotations per minute
s Singlet (nmr)
SDS Sodium dodecyl sulphate
sec Second(s)
T Tense (form or state of enzyme)
t Triplet (in nmr)
TBAHS Tetrabutylammonium hydrogensulfate
TEA Triethanolamine
TIM Triosephosphate isomerase
Tm Melting temperature (of DNA)
TPP Thiamine pyrophosphate or cocarboxylase
Tris Tris(hydroxy)amino methane
Tyr113 Tyr113 residue in L-Ru5P 4-epimerase, coming from the subunit adjacent to the one bearing the active-site Zn²⁺
UDP Uridine 5′-diphosphate
UDP-DH UDP-N-acetylmannosamine dehydrogenase
UDPE UDP-GlcNAc 2-epimerase
UDP-GalNAc UDP-N-acetylgalactosamine
UDP-GlcNAc  Uridine 5'-diphosphate N-acetylglicosamine
UDP-ManNAcUA  UDP-N-acetylmannosaminuronic acid
UDP-ManNAc  Uridine 5'-diphosphate N-acetylmannosamine
UGE  UDP-glucose 4-epimerase
UMP  Uridine monophosphate
UTP  Uridine triphosphate
UV  Ultraviolet
\( v \)  Initial velocity
\( V_{max} \)  Maximum velocity reached with a given enzyme concentration
wecB  Gene encoding UDP-GlcNAc 2-epimerase
WT  Wild-type enzyme
Y229F  L-Ru5P 4-epimerase in which a tyrosine at position 229 has been replaced by a phenylalanine

Standard Abbreviations for Amino Acids

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<th>Code</th>
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**Standard Abreviations for Nucleotides**

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Acknowledgements

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To

All those who always believed in me
1 Enzyme Catalyzed Epimerization of Carbohydrates

1.1 Introduction

In the past decade, the scientific community has become increasingly aware of the significance of carbohydrates in a wide variety of biological events. It is now known that carbohydrates not only serve as energy sources and structural elements, but also play a very important role as signaling molecules. Their involvement in cell-cell recognition has opened up a whole new avenue of research known as glycobiology. One of the main reasons that nature uses them as the key signaling and labeling molecules is that they possess dense functionality and multiple linkage possibilities. Unlike the amino acids, sugar molecules have many chiral centers and an inversion of stereochemistry at one or more of these chiral centers can produce a large number of diastereomers. Further substitutions and oligomerization of these building blocks can generate a wide diversity of structures. Given the numerous carbohydrate building blocks found in nature, it is surprising to see that only a few biosynthetic pathways exist that are capable of generating them from non-carbohydrate precursors. The rest are biosynthesized by modifications to these common “core” units. These modifications include oxidation, deoxygenation, introduction of heteroatoms (such as N or S), chain branching, oligomerization and epimerization.\(^1\) In many cases, these modifications are introduced at the level of the activated sugars, or sugar nucleotides, instead of the free, unactivated sugars.\(^2\) Making new molecules from pre-existing core units is an efficient way that nature has devised to produce a repertoire of sugar molecules without having to create a lengthy biosynthetic pathway for each one of them. Although each of the modifications mentioned above has its own significance,
focus will be on epimerization reactions and the enzymes responsible for catalyzing these reactions.

**1.2 Epimerases and Racemases**

Epimerases and racemases are enzymes that catalyze an inversion of stereochemistry at a stereogenic center in a biological molecule (Fig. 1-1, pg. 2). When a molecule has only one chiral center, an inversion of stereochemistry at that center results in racemization and the enzymes that catalyze such reactions are called racemases (e.g., alanine racemase and mandelate racemase). Since almost all sugar molecules (except glyceraldehyde and dihydroxyacetone) contain more than one chiral center, there are no known sugar racemases. When a molecule has more than one chiral center, an inversion of stereochemistry at one of them results in an epimerization. Enzymes that catalyze such reactions are called epimerases (e.g., D-ribulose-5-phosphate 3-epimerase and UDP-glucose 4-epimerase). Epimerases and racemases are widely distributed in nature. Some epimerases, like UDP-glucose 4-epimerase, are ubiquitous and...
found in organisms ranging from bacteria to human. On the other hand, some epimerases, like D-ribulose-5-phosphate 3-epimerase, are not found in higher animals.

Over the past several decades chemists and biochemists have been interested in the mechanisms by which the epimerases catalyze these seemingly simple reactions. An understanding of their mechanism could lead to the development of their inhibitors and in some cases lead to drug discovery. But in many cases, chemists have been interested in uncovering the details of the mechanistic strategies that these remarkable catalysts employ.

1.3 Mechanisms Employed by Sugar Epimerases

Sugar epimerases may act at the level of monosaccharides or polysaccharides. Irrespective of the nature of the sugar molecule, however, most known epimerases follow certain general mechanistic pathways. These include the simple deprotonation/reprotonation mechanism or the oxidation/reduction mechanism. A few are known to catalyze the reaction by unique mechanisms. The mechanistic aspects of carbohydrate epimerization have been recently reviewed. A short account of the same will be discussed in the following sections along with examples.

1.3.1 Deprotonation/Reprotonation Mechanism

A deprotonation/reprotonation mechanism is employed by enzymes that act at “activated” stereogenic centers. These include stereocenters adjacent to carbonyl groups. The proton at such a center has a relatively low pKₐ (< 30) and hence can be abstracted by a catalytic base in the active site of the enzyme. An initial deprotonation forms a resonance stabilized anion (enolate) (Fig. 1-2, pg. 4). A non-stereospecific reprotonation of the enolate generates the
epimer. Theoretically, two potential strategies can be employed in the deprotonation/reprotonation event – a "one-base" mechanism or a "two-base" mechanism. In a "one-base" mechanism, the deprotonation and reprotonation of both the epimers is achieved with a single, flexible, acid/base residue in the enzyme active site, whereas in a "two-base" mechanism, the epimers are deprotonated and reprotonated using two different catalytic residues. To date no epimerase is known that operates by a one-base deprotonation/reprotonation mechanism.

![Fig. 1-2 Deprotonation/reprotonation mechanism.](image)

Among the carbohydrate epimerases that employ a deprotonation/reprotonation mechanism, the classic example is D-ribulose-5-phosphate 3-epimerase (RPEase, EC 5.1.3.1), which interconverts D-ribulose 5-phosphate (D-Ru5P) and D-xylulose 5-phosphate (D-Xu5P) (Fig. 1-3, pg. 5).\(^5\),\(^6\),\(^7\),\(^8\),\(^9\) In vivo, D-ribulose 5-phosphate and D-xylulose 5-phosphate exist in equilibrium with D-ribose 5-phosphate, and together, the three pentoses form a reversible link between the pentose phosphate pathway and glycolysis. The proposed mechanism of epimerization by RPEase involves deprotonation at C-3, forming an enolate intermediate, followed by reprotonation on the opposite face to give the epimeric product (Fig. 1-3, pg. 5).\(^10\) This mechanism is supported by the observation that solvent-derived deuterium is incorporated at C-3.\(^11\) Moreover, a primary kinetic isotope effect has been observed in the epimerization of
[3-\textsuperscript{2}H]-D-ribulose 5-phosphate, indicating that the C\textsubscript{3}-H bond is broken in the rate-determining step.\textsuperscript{12} On the basis of the crystal structure and substrate modelling with RPEase from potato chloroplast, Asp43 and Asp185 have been proposed to act as the catalytic residues involved in the deprotonation of D-Ru5P and D-Xu5P, respectively.\textsuperscript{13} The assignment of Asp185 as one of the catalytic residues is supported by the fact that the D185N mutant is completely inactive but structurally it is indistinguishable from the wild-type epimerase.\textsuperscript{14}

\textbf{Fig. 1-3 Mechanism of the reaction catalyzed by D-ribulose-5-phosphate 3-epimerase}

Several other epimerases, including tagatose 3-epimerase, mannuronan C-5 epimerase, alginate epimerase, uronate epimerase, GDP-D-mannose 3,5-epimerase-4-reductase (GMER) and dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase (RmlC) are all known to proceed by a simple deprotonation/reprotonation mechanism.\textsuperscript{4}

\textbf{1.3.2 Epimerization at “Unactivated” Centers}

Epimerases that operate at “unactivated” stereocenters cannot employ a direct deprotonation/reprotonation mechanism since the pK\textsubscript{a} of the proton at that center is extremely high. One way in which such enzymes have overcome this difficulty is through the use of a transient oxidation/reduction strategy involving the cofactor NAD\textsuperscript{+} or NADP\textsuperscript{+}. Alternatively, a few examples of enzymes are known that can catalyze the epimerization of unactivated centers
in a cofactor-independent manner. These enzymes must utilize unique mechanistic strategies to bring about the inversion of stereochemistry and hence are very interesting from a mechanistic point of view.

1.3.2.1 Cofactor-dependent Epimerization of “Unactivated” Centers

Fig. 1-4 General mechanism for cofactor-dependent epimerization of an unactivated center

Enzymes using this strategy could either directly oxidize the stereogenic center that is to be inverted or oxidize the carbon adjacent to it (Fig. 1-4, pg. 6). In the first case, the hydride is transferred from the site of inversion to the cofactor and the chiral center is oxidized. Return of the hydride to the opposite face of the carbonyl results in the inversion of stereochemistry (Fig. 1-4, pathway “a”, pg. 6). In the second case, oxidation of the adjacent carbon atom results in the
lowering of $pK_a$ of the hydrogen at the stereogenic center. Consequently, acid/base residues from the enzyme can now promote a non-stereospecific deprotonation/reprotonation at the chiral center, resulting in its inversion. Return of the hydride to the same face of the carbonyl completes the epimerization process (Fig. 1-4, pathway "b", pg. 6).

The most extensively studied enzyme in this category is UDP-galactose 4-epimerase or UDP-glucose 4-epimerase (UGE) (EC 5.1.3.2), which interconverts UDP-glucose and UDP-galactose (Fig. 1-5, pg. 7). This enzyme has been isolated from a variety of sources including *Escherichia coli* (*E. coli*), calf liver, yeast and humans. Most of the structural and mechanistic studies have focused on the epimerase isolated from *E. coli*, which exists as a homodimer with a molecular weight of 79 kDa.\textsuperscript{15}

\begin{center}
\begin{tikzpicture}
\node (A) at (0,0) {UDP-Glucose};
\node (B) at (2,0) {UDP-4-keto-glucose};
\node (C) at (0,-2) {UDP-Galactose};
\node (D) at (2,-2) {UDP-4-keto-glucose};
\node (E) at (1.5,0) {rotation};
\draw[->] (A) edge node[above] {Enz*NAD}$H^+$ (B);
\draw[->] (B) edge node[above] {rotation} (E);
\draw[->] (E) edge node[above] {Enz*NADH} (D);
\draw[->] (D) edge node[above] {Enz} (C);
\node at (1,0) {UDP};
\node at (1,-2) {UDP};
\end{tikzpicture}
\end{center}

*Fig. 1-5 Mechanism of the reaction catalyzed by UDP-glucose 4-epimerase*

Since the initial report of epimerase activity in 1950, a great deal of biochemical research has been directed towards understanding the reaction catalyzed by this enzyme.\textsuperscript{16,17} No incorporation of solvent derived $^2$H or $^3$H or $^{18}$O was observed upon extended incubation of the
enzyme with the substrates, indicating that the reaction does not proceed via a deprotonation/reprotonation or a dehydration/rehydration mechanism.\textsuperscript{18, 19, 20} The enzyme isolated from calf liver showed a requirement for NAD\textsuperscript{+} as an added cofactor, while a tightly bound NAD\textsuperscript{+} was found in the yeast and \textit{E. coli} enzymes.\textsuperscript{21, 22} On incubating the \textit{E. coli} UGE with [4-\textsuperscript{3}H]UDP-glucose for an extended period of time, the radioactivity was found to be distributed between the UDP-hexose and NADH, but no such redistribution was found when [3-\textsuperscript{3}H]UDP-glucose was incubated with the enzyme.\textsuperscript{23} These observations support a mechanism involving direct oxidation/reduction at C-4 and disprove an alternative mechanism involving transient oxidation at C-3 and epimerization via an enolate intermediate. Thus, the first step in the reaction catalyzed by UGE is the abstraction of a proton from the C-4 hydroxyl leading to the transfer of the C-4 hydride to NAD\textsuperscript{+} and formation of UDP-4-ketoglucose and NADH as intermediates (Fig. 1-5, pg. 7). A simple rotation of the sugar moiety of UDP-4-ketohexose about the bond connecting the glycosyl oxygen atom and the β-phosphorus atom in the pyrophosphoryl linkage exposes the opposite face of the keto intermediate to NADH.\textsuperscript{24} The re-addition of the hydride from NADH to the opposite face of the keto-intermediate gives the epimer. The most convincing evidence for the proposed transient oxidation mechanism was the observation that the UDP-4-ketoglucose intermediate is released upon extended incubation of the enzyme with the substrates. This intermediate could be trapped by chemical reduction with NaB\textsubscript{3}H\textsubscript{4} to form [4-\textsuperscript{3}H]UDP-glucose and [4-\textsuperscript{3}H]UDP-galactose.\textsuperscript{25, 26, 27} Furthermore E•NADH, produced by the reduction of E•NAD\textsuperscript{+} with NaBH\textsubscript{4}, could be re-oxidized by treatment with TDP-4-keto-6-deoxyglucose or UDP-4-keto-6-deoxyglucose.\textsuperscript{28} The proposed rotation of the ketohexose intermediate is supported by circular dichroism, fluorescence spectroscopy and X-ray crystallographic studies. Crystal structures of the catalytically inactive S124A/Y229F mutant
with the two substrates show that UDP-glucose and UDP-galactose present opposite faces to the C-4' of the nucleotide.  

UDP-galactose 4-epimerase (UGE) belongs to the superfamily of short chain dehydrogenases/reductases (SDR). Enzymes belonging to this superfamily are typically around 250 amino acid residues in length and contain two signature sequences. The first of these is a YxxxxK motif in which the conserved tyrosine plays a key role in catalysis. The second is a GxxxxGxG motif, which is located in the Rossmann fold that is common to nucleotide binding enzymes. NMR spectroscopy studies on UGE from *E. coli* show that Tyr149 and Lys153 play an important role in increasing the reactivity of the cofactor.  

On the basis of mutagenesis studies, Tyr149 and Ser124 have been implicated in acid/base catalysis, deprotonating and reprotonating the C-4 hydroxyl. These two residues along with Lys153 comprise the Ser, Tyr, Lys triad that is a characteristic of the SDR family members. Kinetically, both the Y149F and S124A mutants were severely compromised as an epimerase (10,000 fold and 3,000 fold, respectively). Moreover, the double mutant Y149F/S124A showed about 7 orders of magnitude lower activity. On the basis of these observations, Tyr149 has been assigned a direct role in deprotonating the C-4 hydroxyl while Ser124 has been assigned a secondary role in catalysis.  

The mechanism of UDP-GlcNAc 4-epimerase and other C-4 epimerases like UDP-xylose 4-epimerase and UDP-glucuronate 4-epimerase are probably similar to that of UGE.  

Two other enzymes that do not act at the C-4 position but are believed to follow an oxidation/reduction mechanism are CDP-tyvelose 2-epimerase, which interconverts CDP-tyvelose and CDP-paratose, and ADP-L-glycero-D-mannoheptose 6-epimerase (AGME), which interconverts ADP-L-glycero-D-mannoheptose and ADP-D-glycero-D-mannoheptose.
The second strategy for cofactor-dependent epimerization is to employ a transient oxidation to acidify the proton at the point of inversion. This is the case with GDP-D-mannose:GDP-L-galactose epimerase, an enzyme that catalyzes a two-fold inversion of stereochemistry at C-3 and C-5 (Fig. 1-6, pg. 10). An NAD⁺ mediated oxidation of C-4 results in the lowering of the pKₐ of the protons at C-3 and C-5. An enzymic base-catalyzed inversion of stereochemistry by simple deprotonation/reprotonation at both C-3 and C-5 followed by a stereospecific reduction of C-4 gives the product. Experimentally, this mechanism can be distinguished from the direct oxidation/reduction at the site of inversion by the fact that when inversion occurs through the formation of an enol(ate) intermediate, solvent isotope is incorporated at the site of epimerization. In the GDP-D-mannose:GDP-L-galactose epimerase reaction, inversions of stereochemistry at C-3 and C-5 occur with incorporation of solvent derived tritium at both positions, implying that they proceed via ene-diol(ate) intermediates.
1.3.2.2 Cofactor-independent Epimerization of “Unactivated” Centers

Enzymes catalyzing the epimerization of an “unactivated” center in a cofactor-independent manner must utilize unique mechanistic strategies to bring about the inversion of stereochemistry. Only two examples are known of enzymes that catalyze such reactions: L-ribulose-5-phosphate 4-epimerase and UDP-N-acetylglucosamine 2-epimerase.

(a) L-Ribulose-5-Phosphate 4-Epimerase

Fig. 1-7 Conversion of L-arabinose to L-ribulose-5-phosphate by the products of the araBAD operon. A). The arabinose operon with genes araCBAD. \( p_C \) and \( p_{BAD} \) are the promoters for the araC and araBAD operons, respectively. The \( araC \) gene product regulates the promoters for \( araC \) (\( p_C \)) and \( araBAD \) (\( p_{BAD} \)). B). The reactions catalyzed by the products of \( araBAD \) operon.

L-Ribulose-5-phosphate 4-epimerase (L-Ru5P 4-epimerase, EC 5.1.3.4) is a bacterial enzyme that catalyzes the interconversion of L-ribulose 5-phosphate (L-Ru5P) and D-xylulose 5-
phosphate (D-Xu5P) (Fig. 1-7, pg. 11). The epimerase is one of the three gene products of the arabinose operon (araBAD). An operon is a set of genes whose expression is controlled by the same promoter. The products of the araBAD operon, L-arabinose isomerase (araA), L-ribulokinase (araB) and the L-ribulose-5-phosphate 4-epimerase (araD), allow the bacteria to use the pentose sugar, L-arabinose, as a carbon source by converting it into D-Xu5P, which can then enter the pentose phosphate pathway (Fig. 1-7, pg. 11). L-Arabinose is found in plant pectins, gums and complex polysaccharides. Production of these enzymes is induced in E. coli, Lactobacillus plantarum and Bacillus subtilis by growth on L-arabinose and in Aerobacter aerogenes by growth on L-arabinose, L-arabitol or L-xylose. In E. coli, in the absence of L-arabinose, the dimeric AraC protein actively represses its own synthesis and the synthesis of the AraB, AraA, and AraD gene products. It is interesting to note here that L-Ru5P 4-epimerase and D-Ru5P 3-epimerase convert the epimers L-Ru5P and D-Ru5P, respectively, into D-Xu5P. This is an excellent example of stereospecific recognition by enzymes.
Fig. 1-8 Proposed mechanisms for the reaction catalyzed by L-ribulose-5-phosphate 4-epimerase. (A) Dehydration/rehydration mechanism (B) Retroaldol/aldol mechanism

The *E. coli* L-ribulose-5-phosphate 4-epimerase exists as a homotetramer with a molecular mass of 25.5 kDa (231 amino acid residues) per subunit and requires a divalent metal ion for catalysis. The enzyme is inactivated by treatment with EDTA and is reactivated to varying extents by the addition of divalent metal ions in the order: Mn$^{2+} > $ Co$^{2+} > $ Ni$^{2+} > $ Ca$^{2+} > $ Zn$^{2+} > $ Mg$^{2+}$. Previous studies have confirmed that the L-Ru5P 4-epimerase does not require NAD$^+$ or any other electron acceptor for activity. Furthermore, no primary kinetic isotope effect was observed during the epimerization of [4-$^3$H]-D-xylulose-5-phosphate, demonstrating that cleavage of the C$_4$-H bond is not a rate-determining step. These observations
rule out all the mechanisms previously discussed in this chapter. Instead, two other mechanisms were proposed—a dehydration/rehydration mechanism across C-3/C-4 and a retroaldol/aldol mechanism. The dehydration/rehydration mechanism (Fig. 1-8A, pg. 13) involves an initial deprotonation at C-3 followed by the elimination of the C-4 hydroxyl to give an enone intermediate. The hydroxyl must then re-add to the opposite face of the enone intermediate with protonation occurring on the same face. However, previous experiments showed that solvent derived $^3$H or $^{18}$O is not incorporated into the products, and [4-$^3$H]D-xylulose-5-phosphate does not lose its label during epimerization. The observed lack of solvent isotope incorporation requires that neither the hydroxyl nor the proton exchange with solvent during the lifetime of the intermediate. The alternate mechanism proposed is the retroaldol/aldol mechanism (Fig. 1-8B, pg. 13). It involves deprotonation of the C-4 hydroxyl, initiating a retroaldol cleavage between C-3 and C-4, and the formation of a metal-bound dihydroxyacetone enolate and glycolaldehyde phosphate as intermediates. A re-orientation then occurs (possibly a simple bond rotation) that exposes the opposite face of the aldehyde to the same face of the enolate. Finally, an aldol addition generates the epimeric product.
Fig. 1-9 Reactions catalyzed by L-fuculose-1-phosphate aldolase and L-rhamnulose-1-phosphate aldolase

Indirect evidence supporting the retroaldol/aldol mechanism comes from a 26% sequence identity with the metal-dependent class-II aldolase, L-fuculose-1-phosphate aldolase (FucA, EC 4.1.2.17). The aldolase catalyzes the reversible cleavage of L-fuculose 1-phosphate (Fuc1P) to dihydroxyacetone phosphate (DHAP) and L-lactaldehyde (Fig. 1-9, pg. 15). The aldolase is highly stereospecific. The cleavage of the C-4 epimer of L-fuculose 1-phosphate, L-rhamnulose 1-phosphate, is catalyzed by a different enzyme, L-rhamnulose-1-phosphate aldolase (Fig. 1-9, pg. 15). The mechanism proposed for L-fuculose-1-phosphate aldolase (in the cleavage direction) involves an initial deprotonation of the C-4 hydroxyl that promotes a retroaldol cleavage, followed by the protonation of the dihydroxyacetone phosphate enolate intermediate to give the products (Fig. 1-9, pg. 15). Analysis of crystal structures of the
aldolase with and without a bound phosphoglycolohydroxamate inhibitor, as well as site-directed mutagenesis studies have identified Glu73 as the catalytic acid/base residue involved in both deprotonating the C-4 hydroxyl and protonating the enolate intermediate. Furthermore, Glu73, along with His92, His94 and His155, has been implicated in the binding of the divalent metal in the absence of the substrate. It has been proposed that upon substrate binding, Glu73 is displaced from the metal and then acts as the catalytic residue. By an analysis of the sequence homology, the corresponding residues in L-Ru5P 4-epimerase are Asp76, His95, His97 and His171. Mutagenesis studies have shown that, indeed, His95 and His97 are involved in metal binding in the epimerase, strengthening the notion that the aldolase and the epimerase share a common fold. However, the first direct evidence for the retroaldol/aldol mechanism for L-Ru5P 4-epimerase came from the observation that the epimerase was able to catalyze a slow aldol condensation between dihydroxyacetone and glycolaldehyde phosphate to give the pentose phosphates. This establishes that the epimerase active site is capable of forming and cleaving carbon – carbon bonds.

At the same time as the work described in this thesis was being completed, two papers were published by Cleland et al. One reported on the $^2$H and $^{13}$C kinetic isotope effect studies and the other on the role of metal ions in catalysis by L-Ru5P 4-epimerase. Primary $^{13}$C kinetic isotope effects were observed at both C-3 and C-4 during the epimerization of L-Ru5P with the wild-type (WT) epimerase. In addition, no primary deuterium isotope effects were detected at either position. These observations rule out the dehydration/rehydration mechanism and support the retroaldol/aldol mechanism. Cleland and co-workers also mutated the nine tyrosine residues in the enzyme into phenylalanine and two of the histidines into asparagines (H97N and H95N) and screened them for activity. The Y229F showed 1500-fold lower activity as compared to the WT epimerase. The H97N was only slightly slower (68%) than the WT.
While the WT and the Y229F mutant retained their metal during the purification process, the two histidine mutants tended to lose their metal ions, suggesting that these histidine residues are required for metal binding. The CD spectra of these enzymes, with and without the substrate, showed that while the WT and Y229F mutant were structurally similar and showed similar structural changes on the addition of substrate, the two histidine mutants were similar to each other but different from the WT and Y229F in both cases. In all four enzymes, the CD spectra of the apoenzyme (without a bound Zn$^{2+}$) and the Zn$^{2+}$-substituted enzyme were similar, indicating that the metal only plays a catalytic role and not a structural role in the active site. The lack of change in the CD spectra of the WT apoenzyme on substrate addition indicated that the metal bound to the enzyme before the substrate. Furthermore, it was observed that the C-terminus mutant Y229F showed a 1000-fold decrease in the value of $k_{\text{cat}}$ while the $K_m$ values did not change much, implying that this residue acts as one of the catalytic residues in the epimerization reaction. On the basis of kinetic and spectral studies it was concluded that the Tyr 229 is not a metal ligand but is probably involved in protonating/deprotonating the C-4 hydroxyl of one of the substrates. His95 and His97 were each assigned the role of a metal binding ligand. Cleland et al. were able to measure the $K_m$ value of various metal ions and showed that they decrease in the order Mg$^{2+}$ > Mn$^{2+}$ > Co$^{2+}$ > Zn$^{2+}$. Furthermore, EPR studies of the Mn$^{2+}$ substituted enzyme showed that the enzyme-bound Mn$^{2+}$ has an octahedral geometry (co-ordination number = six) and is hydrated by three water molecules. This indicates that there are three metal-binding ligands in the enzyme, two of which are His95 and His97.
(b) UDP-\(N\)-Acetylglucosamine 2-Epimerase

Fig. 1-10 Reactions catalyzed by UDP-GlcNAc 2-epimerase (shown in dotted box) and UDP-ManNAc dehydrogenase

A second enzyme that catalyzes a cofactor-independent epimerization of an unactivated stereocenter is UDP-\(N\)-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase or UDPE) (EC 5.1.3.14). It is commonly found in bacteria and interconverts UDP-\(N\)-acetylglucosamine (UDP-GlcNAc) and UDP-\(N\)-acetylmannosamine (UDP-ManNAc) (Fig. 1-10, pg. 18). The UDP-ManNAc is then oxidized by UDP-ManNAc dehydrogenase to give UDP-\(N\)-acetylmannosaminuronic acid (UDP-ManNAcUA). The two enzymes together provide the bacteria with activated forms of ManNAc and ManNAcUA.\(^\text{66}\) ManNAc is found in the linkage unit of the cell wall of most gram-positive bacteria. The linkage unit connects the teichoic acid and teichuronic acid molecules to the peptidoglycan (please refer to Appendix-1 for more details). It is also found in the anti-phagocytic capsular polysaccharide in pathogenic strains of bacteria such as *Streptococcus pneumoniae* types 19F and 19A.\(^\text{67, 68}\) ManNAcUA is used in a cell surface polysaccharide, teichuronic acid of *Micrococcus lysodeicticus*, in the K7 and K56 antigens on the capsule of some *E. coli*, in the K15 antigen of *Vibrio parahaemolyticus* and is a constituent of the enterobacterial common antigen found in gram-negative bacteria of the enterobacteriaceae family (Appendix-1).\(^\text{66}\)
The gene encoding the epimerase (nfrC or wecB or rffE) has been cloned and over-expressed in *E. coli*. UDP-GlcNAc 2-epimerase from *E. coli* exists as a homodimer with a subunit molecular mass of 42.2 kDa and shows an absolute requirement for UDP-GlcNAc for activity. The rate of epimerization (UDP-GlcNAc → UDP-ManNAc) as a function of UDP-GlcNAc concentration shows sigmoidicity with a Hill coefficient of 2.0. The reaction in the reverse direction (UDP-ManNAc → UDP-GlcNAc) is not catalyzed by the enzyme unless a small amount of activating UDP-GlcNAc is present in the reaction mixture. It has been suggested that either a modulator site is present on the enzyme or the active site of one subunit must be occupied by UDP-GlcNAc in order for the other subunit to function.

**Mechanism A**

\[
\text{UDP-GlcNAc} \quad \text{NAD}^+ \rightarrow \text{NADH} \quad \text{Enz} \quad \text{UDP-ManNAc}
\]

**Mechanism B**

\[
\text{UDP-GlcNAc} \quad \text{NAD}^+ \rightarrow \text{NADH} \quad \text{Enz} \quad \text{UDP-ManNAc}
\]

Fig. 1-11 Proposed mechanisms for the reaction catalyzed by UDP-GlcNAc 2-epimerase
Initial studies showed that the activity of the purified enzyme was not affected by the addition of cofactors such as NAD\(^+\); however, the possibility of a tightly bound cofactor (as in *E. coli* UDP-glucose 4-epimerase discussed in section 1.3.2.1, pg. 7) could not be ruled out. It was also found that a solvent-derived proton is incorporated at C-2 during the epimerization process.\(^{71}\) This observation rules out a direct oxidation/reduction mechanism. An alternate mechanism (Fig. 1-11, Mechanism A, pg. 19) was proposed by Salo *et al.* involving an NAD\(^+\)-dependent oxidation at C-3, followed by epimerization via enolization (similar to the mechanism of GDP-D-mannose:GDP-L-galactose epimerase discussed in Section 1.3.2.1, pg. 10).\(^{72}\) Stereospecific reduction at C-3 gives the product and regenerates NAD\(^+\). However, when the enzyme was eventually cloned and overexpressed, it was possible to demonstrate the absence of any stoichiometric amounts of bound cofactor.\(^{69}\) Thus, any mechanism involving transient oxidation could be ruled out. More recent studies have shown that the epimerization of [2-\(^2\)H]UDP-GlcNAc is slowed by a primary kinetic isotope effect of 1.8 on the value of \(k_{cat}\), indicating that the C-H bond cleavage is at least partially rate determining.\(^{73}\) Moreover, when UDP-GlcNAc with an \(^{18}\)O-label selectively incorporated at the anomeric position of the GlcNAc moiety was incubated with the epimerase, scrambling of the \(^{18}\)O label into both the “bridging” (anomeric) and “non-bridging” phosphate positions in a statistical ratio was observed (Fig. 1-12, pg. 21).\(^{74}\) This is consistent with a mechanism where the anomeric C-O bond cleaves, generating an enzyme-bound UDP intermediate (Fig. 1-11, Mechanism B, pg. 19). Rotation of the single bonds between \(\alpha\)- and \(\beta\)-phosphates of UDP allow the label to equilibrate into all three equivalent positions. Reformation of the anomeric C-O bond results in the positional scrambling of the isotope. On the basis of these observations a mechanism lacking the requirement for NAD\(^+\) was proposed. In the UDP-GlcNAc to UDP-ManNAc direction, abstraction of the C-2 proton by an enzymatic base initiates an *anti*-elimination of UDP and the
formation of 2-acetamidoglucal as an intermediate. A subsequent syn-addition of UDP and a proton on to 2-acetamidoglucal gives the epimeric product. This mechanism is also supported by the observation that the intermediates (UDP and glycal) are released upon extended incubation of the enzyme with UDP-GlcNAc. The intermediates, being thermodynamically more stable than the substrates, accumulate over time. A detailed mechanism of how the syn- and anti- eliminations of UDP are catalyzed in mechanism B remains unclear. An E1cb mechanism can be ruled out since the pKₐ of the proton at C-2 is very high. Instead, an E2 or E1 mechanism could be at play. The simple mechanism B outlined above may be more complicated by the involvement of covalent catalysis with enzyme-bound intermediates and/or by the possibility of neighboring group participation by the acetamido functionality.

![Diagram of UDP-GlcNAc 2-epimerase](image)

**Fig. 1-12 Positional isotope exchange in UDP-GlcNAc 2-epimerase**

Recently, the structure of UDP-GlcNAc 2-epimerase from *E. coli* with a bound UDP has been solved to 2.5 Å by X-ray crystallography. Although the enzyme was crystallized in the presence of UDP-GlcNAc, in the final structure only the UDP moiety is clearly visible in both subunits of the dimer. Either the sugar portion of the substrate is disordered, or the intermediates were formed during crystallization and the glycal is disordered or has diffused out of the active
Each subunit of the homodimeric epimerase is composed of two topologically similar domains with a deep cleft at the domain interface (Fig. 1-13, pg. 22). Both, N- and C-terminal domains contain a core structure of parallel β-sheets connected by α-helices. Such a fold is commonly referred to as the Rossmann fold and is generally associated with dinucleotide binding. The two domains are joined through two α-helices (residues 171 – 205) and the substrate is bound at the interdomain cleft.

Fig. 1-13 Structure of UDP-GlcNAc 2-epimerase with bound UDP
Interestingly, the two subunits show conformations that differ by an interdomain rotation of about 10° within a given subunit. Thus, the active site of one subunit is "closed" while that of the other is "open" (Fig. 1-13, pg. 22). This conformational difference between the subunits most likely plays an important role in the substrate cooperativity observed in the epimerase. Interestingly, only a single substrate/effector binding site was observed in each subunit. One of the proposed mechanisms for allostery in UDPE is that binding of UDP-GlcNAc to one subunit induces a conformational change across the dimer interface causing a change in substrate affinity and/or catalytic efficiency in the vacant site. Thus, at any point in time only one subunit is catalytically active. This would, therefore, be a unique case of "half-site reactivity". In enzymes showing the phenomenon of half-site reactivity, their reaction with a substrate or substrate analogue shows a stoichiometry equal to one-half the number of "identical" subunits.

![Diagram of reaction catalyzed by β-glucosyltransferase (BGT) and glycogen phosphorylase (GP)](Fig. 1-14 Reactions catalyzed by β-glucosyltransferase (BGT) and glycogen phosphorylase (GP))
An interesting feature of the structural work is that the epimerase shows significant structural homology (but <10% sequence homology) to a superfamily of glycosyltransferases that includes glycogen phosphorylase (GP), \(^{77}\) MurG, \(^{78}\) UDP-glucosyltransferase GtfB \(^{79}\) and the DNA modifying \(\beta\)-glucosyltransferase (BGT) of T4 bacteriophage.\(^{80, 81, 82}\) BGT catalyzes the glycosylation of DNA by transferring the glucose moiety from UDP-glucose to hydroxymethylated cytosine, while GP catalyzes the cleavage of a glucosyl moiety from glycogen to give glucose 1-phosphate (Fig. 1-14, pg. 23). MurG catalyzes the transfer of GlcNAc from UDP-GlcNAc to the C-4 hydroxyl of a lipid-linked \(N\)-acetylmuramoyl pentapeptide. The glucosyltransferase GtfB transfers a glucose moiety from UDP-glucose to the vancomycin aglycone. Thus, all these enzymes are involved in the formation and/or cleavage of phosphoglycosyl bonds and their reactions have been proposed to proceed via oxocarbenium-like transition states.\(^{83}\) The structural similarity with UDP-GlcNAc 2-epimerase could be an indication of their common mechanism and, thus, their evolutionary link. Interestingly, an interdomain rotation of \(\sim 5^\circ\), similar to that in UDPE, has been found in both BGT and GP.\(^{77}\) In BGT, which is a monomeric enzyme, the interdomain rotation occurs on binding of UDP-glucose and results in conversion of the "open" conformation to the "closed" conformation. In GP, the interdomain rotation is related to the transition between the high activity R-state and the low activity T-state upon binding of the effector AMP or upon phosphorylation.
Fig. 1-15 (A) Reaction catalyzed by mammalian UDP-GlcNAc 2-epimerase/ManNAc kinase. Reaction catalyzed by the epimerase domain has been shown in a dashed-box. (B) Proposed mechanism of the epimerization reaction

The mammalian counterpart of the bacterial UDP-GlcNAc 2-epimerase is a bifunctional UDP-GlcNAc 2-epimerase/ManNAc 6-kinase that converts UDP-GlcNAc into UDP and ManNAc 6-phosphate (Fig. 1-15A, pg. 25). The epimerase activity converts UDP-GlcNAc irreversibly into UDP and ManNAc and the kinase then phosphorylates the latter to give ManNAc 6-phosphate. The first step is not a true epimerization as hydrolysis of the sugar-UDP bond accompanies the inversion of stereochemistry at C-2. The N-terminal half of the mammalian epimerase/kinase (residues 1 – 376) shows about 22% sequence identity with the *E. coli* UDP-GlcNAc 2-epimerase while the C-terminal half (residues 410 – 684) shows sequence homology to kinases (31% sequence identity with glucokinase from *Streptomyces coelicolor*).
The mechanism employed by the mammalian epimerase has been proposed to be similar to that of the \textit{E. coli} epimerase. UDP is a non-competitive inhibitor of the enzyme indicating an ordered mechanism, with UDP being released first, followed by ManNAc.\textsuperscript{88} Moreover, the epimerization reaction proceeds with the incorporation of a solvent derived tritium at C-2.\textsuperscript{89} This indicates that the epimerization of UDP-GlcNAc into ManNAc in the mammalian enzyme also proceeds via removal of the C-2 proton. It has been shown that UDP-ManNAc is not an intermediate, although the enzyme does accept it as an alternate substrate and hydrolyzes it to form UDP and ManNAc. No UDP-GlcNAc is observed during this conversion and the rate of hydrolysis is much slower than that seen with UDP-GlcNAc. On the basis of the above observations, a two step mechanism has been proposed for the mammalian epimerase (Fig. 1-15B, pg. 25). The first step involves the elimination of UDP from UDP-GlcNAc to give a 2-acetamidoglucal intermediate, and the second involves the hydration of 2-acetamidoglucal to give ManNAc.\textsuperscript{88, 90} The mechanism involving 2-acetamidoglucal as the intermediate is supported by the fact that synthetic 2-acetamidoglucal is converted to ManNAc by the enzyme whereas its spontaneous hydration in solution gives GlcNAc.\textsuperscript{90}

A unique feature of the mammalian enzyme is that it is allosterically inhibited by CMP-\textit{N}-acetylneuraminic acid (CMP-Neu5Ac).\textsuperscript{90} A plot of the concentration of CMP-\textit{N}-acetylneuraminic acid against percentage inhibition gives a sigmoidal plot with a Hill coefficient of 4.0. CMP-Neu5Ac at 0.1 mM inhibits the epimerase activity completely while 0.1 mM \textit{N}-acetylneuraminic acid does not influence the activity at all. The negative allosteric control of the mammalian epimerase by the activated sialic acid, CMP-Neu5Ac, might have biological significance since the reaction catalyzed by this enzyme has been found to be the rate determining step in the sialic acid biosynthesis pathway.\textsuperscript{91} CMP-Neu5Ac is the final product in this pathway. The \textit{N}-acetylneuraminic acid from CMP-Neu5Ac is then transferred to a variety of
cell surface oligosaccharides to form sialylated glycoconjugates. Sialylated glycoconjugates are often present as labelling molecules on cell surfaces and play an important role in many biological processes, including cell-adhesion, metastasis and signal transduction.

1.4 Aims of the Thesis

As discussed above, there are only two known carbohydrate epimerases that catalyze the cofactor-independent inversion of stereochemistry at an “unactivated” stereogenic center. These are L-ribulose-5-phosphate 4-epimerase and UDP-GlcNAc 2-epimerase. Previous work done on these enzymes has shed light on the unique chemistry employed by these enzymes. However, detailed mechanisms of how the enzymes catalyze these reactions within their active sites are still unknown.

L-Ribulose-5-phosphate 4-epimerase and L-fuculose-1-phosphate aldolase both employ mechanism involving C-C bond cleavage/formation. These two enzymes also share 26% sequence homology. However their substrates are different and the reaction catalyzed by the two enzymes are also different. Structural and mutagenesis studies done on the aldolase have shown that it uses a single amino acid residue, Glu73, for deprotonation and reprotonation of the substrate and intermediate, respectively. However, Asp76, which is the residue homologous to Glu73 of the aldolase, is not a key catalytic residue in the epimerase. This leaves us with questions about the identity of the catalytic residues in L-ribulose-5-phosphate 4-epimerase.

The substrates of the epimerase and aldolase differ in the relative position of their carbonyl and phosphate groups. The carbonyl group coordinates with the metal and the phosphate group is one of the main anchoring groups in the substrate. Previous studies have shown that the metal-binding ligands have been conserved between the epimerase and the
aldolase. According to sequence homology, the phosphate-binding residues of the aldolase have also been conserved in the epimerase. Site-directed mutagenesis studies could be used to further investigate the location of the phosphate binding pocket in the epimerase.

Another aspect worth investigating is whether the epimerase, which shows residual aldolase activity, could be converted into a better aldolase by a single mutation. The key difference in the mechanistic steps of the epimerase and the aldolase is that in the former, the enolate intermediate adds back to the aldehyde, while in the latter, the enolate intermediate is protonated and released into solution. The residue responsible for protonating the enolate in the aldolase is Glu73. The corresponding residue in the epimerase, Asp76, is one carbon shorter and might be farther from the enolate. A mutation of Asp76 into a glutamate might provide the enzyme with a residue for protonating the enolate and thereby, convert the epimerase into a better aldolase.

In the case of UDPE, the structure of the enzyme crystallized in the presence of substrate is known. However, the sugar portion of the substrate is not visible in the final structure. On the basis of sequence homology with UDPE from other sources and the location of the conserved residues within the active site, five residues have been identified as possible catalytic residues. Mutagenesis studies on these residues could help identify the catalytic residues involved in deprotonating C-2 of UDP-GlcNAc and UDP-ManNAc.

UDPE is allosterically regulated by one of its substrates, UDP-GlcNAc. This is a unique case in that UDPE catalyzes a unimolecular reaction and the substrate itself is the regulator. No other effector molecules are known for this enzyme. The enzyme is a homodimer with no separate regulatory site visible in the crystal structure. A combination of site-directed
mutagenesis, kinetics and binding studies could be used to determine the extent and the nature of allosteric regulation in UDPE.
2 L-Ribulose-5-Phosphate 4-Epimerase

L-Ribulose-5-phosphate 4-epimerase (L-Ru5P 4-epimerase) is a bacterial enzyme that interconverts L-ribulose 5-phosphate and D-xylulose 5-phosphate. It catalyzes this reaction via a retroaldol/aldol mechanism and is believed to be part of a superfamily of aldolases/epimerases. L-Ru5P 4-epimerase shows 26% sequence homology to a metal-dependent class-II aldolase, L-fuculose-1-phosphate aldolase (L-Fu1P aldolase), which catalyzes a C-C bond cleavage between C-3 and C-4 of L-fuculose 1-phosphate to give L-lactaldehyde and dihydroxyacetone phosphate (DHAP) (Fig. 2-1, pg. 30).

Mechanistically, the epimerase and the aldolase are very similar. In both enzymes, starting from the pentose sugar phosphate, the C-4 hydroxyl is deprotonated and an enolate and
aldehyde are formed as intermediates. In the epimerase, the enolate intermediate adds back to
the opposite face of the aldehyde to give the C-4 epimer, while in the aldolase, the enolate
intermediate is protonated and released into solution (Fig. 2-1, pg. 30). (Please refer to section
1.3.2.2(a), pg. 11 for more details).

Questions that remain to be answered with the L-Ru5P 4-epimerase include the nature of
the catalytic residues involved in deprotonating/reprotonating the C-4 hydroxy! of the two
epimers, and the identification of substrate binding residues and how they compare with those of
the aldolase. Here, we have used a combination of site-directed mutagenesis, kinetic studies and
crystallography to identify one of the potential catalytic residues involved in deprotonating the
C-4 hydroxyl of D-Xu5P. We have also shown that the phosphate binding pocket in the aldolase
has been conserved in the epimerase. An attempt was also made to convert the epimerase into an
aldolase through a single mutation.

2.1 Previous Work Done

The E. coli epimerase exists as a homotetramer with a molecular mass of 25.5 kDa per
subunit and requires a divalent metal ion for catalysis. Initial work done on this enzyme
showed that it is inactivated by treatment with EDTA and is reactivated to varying extents by
the addition of divalent metal ions in the order: Mn^{2+} > Co^{2+} > Ni^{2+} > Ca^{2+} > Zn^{2+} > Mg^{2+}. It was also shown that the epimerase does not require NAD^{+} or any other electron acceptor
for activity. Kinetic and site-directed mutagenesis studies on the recombinant enzyme showed
that it catalyzes the epimerization reaction via a retroaldol/aldol mechanism (Fig. 2-1, pg. 30).
Indirect evidence for this mechanism comes from a 26% sequence identity with the class-II
aldolase, L-fuculose-1-phosphate aldolase, which also uses a divalent metal ion for catalysis. L-
Fuculose-1-phosphate aldolase catalyzes the reversible cleavage of L-fuculose-1-phosphate
(Fuc1P) to dihydroxyacetone phosphate (DHAP) and L-lactaldehyde. Johnson et al. showed that the epimerase and the aldolase share common metal binding ligands His95 and His97. They were also able to show that the 4-epimerase is capable of catalyzing the C-C bond formation between dihydroxyacetone (DHA) and glycolaldehyde phosphate (GAP) to give the two epimers L-Ru5P and D-Xu5P.

In the case of the aldolase, it has been shown that Glu73 coordinates with the metal in the absence of substrate, but on substrate binding this residue moves away from the metal and acts as the catalytic residue. On the basis of the crystal structure and site-directed mutagenesis studies on the L-Fu1P aldolase, it has been suggested that Glu73 is the residue responsible for deprotonating the C-4 hydroxyl of L-Fu1P and for protonating the enolate intermediate. The epimerase residue that is homologous to Glu73 of the aldolase is Asp76. Johnson et al. mutated Asp76 into an asparagine and showed that it is not the catalytic residue in the epimerase.

Fig. 2-2 Subunit structure of L-Ru5P 4-epimerase and L-Fu1P aldolase
During the early stages of this work, L-Ru5P 4-epimerase was crystallized and the structure solved by molecular replacement using L-Fu1P aldolase as the model. Interestingly, the three-dimensional structure of the epimerase shows a very close similarity to that of the aldolase (over 192 out of a maximum of 208 Cα pairs, RMSD = 1.5 Å) (Fig. 2-2, pg. 32). Both enzymes exist as a homotetramer with the active site located in the inter-subunit region. The last eight residues of the C-terminal region are not visible in the crystal structure of either of the enzymes. Sequence homology and mutagenesis studies had previously shown that three histidine residues act as the metal binding ligands in both the epimerase and the aldolase. This was confirmed by the X-ray crystallography data. In the aldolase, Glu73 forms the fourth ligand that coordinates with the metal in the absence of bound substrate. In the epimerase, Asp76, which has a shorter side-chain, is farther away from the metal and cannot act as the metal-binding ligand. However, considering steric and geometric constraints, it has been suggested that there could be two water molecules, one coordinated between the side-chain of Asp76 and Zn²⁺, and one coordinated to the solvent-exposed face of the Zn²⁺.

2.2 Enzyme Kinetics

Enzymes are proteins that catalyze chemical reactions within living cells. Catalysts speed up the rate of a reaction without themselves undergoing any net change and without affecting the equilibrium constant of the reaction. This is achieved by lowering the energy of activation for the reaction. In cases where it is possible to compare non-enzymatic and enzymatic rates, it has been found that enzymes enhance reaction rates by factors of up to 10¹⁵. However the property that sets enzymes apart from any other known catalyst is their specificity. Enzymes exhibit a high degree of specificity for their substrates and/or the type of reaction catalyzed. It was this aspect of enzymes that intrigued scientists since the early 19th century.
Enzyme kinetics is a branch of enzymology that deals with the factors that affect the rate of enzyme-catalyzed reactions. These include factors such as the concentration of enzymes and ligands (substrates, products, inhibitors, effectors, etc), pH, ionic strength and temperature. A brief overview of enzyme kinetics relevant to this thesis is presented here with emphasis on the significance of kinetic constants. 

(i) **Henri-Michaelis-Menten equation**

In 1902, A. J. Brown proposed that enzyme (E) and substrate (S) first combine to form an enzyme-substrate complex (ES), which then dissociates into enzyme and product (P).

\[ E + S \rightleftharpoons_{K_m} ES \rightarrow E + P \]  

The existence of an enzyme-substrate complex was inferred from (a) the high degree of specificity exhibited by enzymes, (b) the shape of the velocity versus substrate concentration curve, and (c) the fact that substrates frequently protected enzymes from inactivation. In 1903, V. Henri derived the first general rate equation for enzyme catalyzed reactions. Ten years later L. Michaelis and M. L. Menten confirmed Henri’s experimental work and presented a slightly modified version of the rate equation which came to be known as the Henri-Michaelis-Menten equation. The derivation of the equation was based on the following assumptions:

1. The enzyme is a catalyst (proposed in 1835 to 1837 by Berzelius).

2. The enzyme and substrate react rapidly to form an enzyme-substrate complex (proposed in 1902 by Brown).

3. Only a single substrate and a single enzyme-substrate complex are involved and the enzyme-substrate complex breaks down directly to form free enzyme and product.
4. Enzyme, substrate and the enzyme-substrate complex are at equilibrium; that is, the rate at which ES dissociates to E + S is much faster than the rate at which ES breaks down to form E + P (rapid equilibrium assumption).

5. The substrate concentration is much larger than the enzyme concentration so that the formation of the ES complex does not alter the substrate concentration.

6. The overall rate of the reaction is limited by the breakdown of the ES complex to form free enzyme and product.

7. The velocity is measured during the early stages of the reaction (initial velocity conditions) so that the reverse reaction is insignificant.

The overall enzyme catalyzed reaction is given by:

\[
E + S \xrightleftharpoons[k_{-1}]{k_1} ES \xrightarrow[k_{cat}]{} E + P
\]  

(2.2)

The Henri-Michaelis-Menten equation is given by:

\[
v = \frac{k_{cat} [E] [S]}{K_S + [S]}
\]  

(2.3)

or

\[
v = \frac{V_{max} [S]}{K_S + [S]}
\]  

(2.4)

where \( v \) = initial velocity at the given substrate concentration.

\( V_{max} = k_{cat} [E]_t \) = limiting maximal velocity that would be observed when all the enzyme is present as ES complex

\([S] = \) a fixed substrate concentration
\[ K_S = \text{dissociation constant of the ES complex.} = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]} \]

\[ [E]_t = \text{total enzyme concentration} = [E] + [ES] \]

The \([E]_t\) in the above equation represents the concentration of catalytic sites. If the enzyme contains one catalytic site per molecule, then \([E]_t\) is the molar concentration of the enzyme. However, if the enzyme contains multiple identical subunits with independent catalytic sites then \([E]_t\) represents the molar concentration of subunits. While doing enzyme assays the initial velocity conditions are maintained by measuring the rate of the reaction when less than 5% of the total substrate has been consumed.

The constant \(k_{\text{cat}}\) is called the turnover number, molecular activity, or catalytic rate constant. It represents the moles of product formed per unit time per mole of the enzyme (or catalytic site) and may have values ranging from 50 to \(10^7\) min\(^{-1}\). The reciprocal of \(k_{\text{cat}}\) represents the time required to complete one catalytic cycle. The significance of \(k_{\text{cat}}\) is that it allows us to calculate the maximal velocity at which an enzymatic reaction can proceed at a fixed enzyme concentration and at infinite substrate concentration (\(V_{\text{max}}\)). Further, since \(k_{\text{cat}}\) relates to the chemical steps subsequent to the formation of the ES complex, changes in \(k_{\text{cat}}\) brought about by changes in the enzyme (e.g., mutagenesis of specific amino acid residues), solution conditions (e.g., pH, ionic strength, temperature, etc.), or substrate identity (e.g., structural analogues or isotopically labelled substrates) define perturbations that affect the chemical steps in enzymatic catalysis. Thus, a decrease in \(k_{\text{cat}}\) of more than one or two orders of magnitude following mutagenesis of a specific amino acid residue could imply that that particular residue plays an important role in one of the chemical steps in the reaction. However, since \(k_{\text{cat}}\) reflects multiple chemical steps, it does not provide detailed information on the rates
of any of the individual steps subsequent to substrate binding. Instead, it provides a lower limit on the first-order rate constant of the rate-determining step following substrate binding.

(ii) Steady state model of enzyme kinetics or Briggs-Haldane equation

The Henri-Michaelis-Menten equation is derived with the assumption that E, S and ES are in rapid equilibrium, i.e., \( k_{-1} \gg k_{\text{cat}} \) (equation 2.2). However, when \( k_{\text{cat}} \) is comparable to or larger than \( k_{-1} \), within a short period after starting the reaction, ES would build up to a near-constant or “steady-state” level which would be lower than the equilibrium concentration of ES. In such cases the rate of the reaction will be proportional to the steady-state concentration of ES and not its equilibrium concentration. The Briggs-Haldane equation derived under steady-state conditions is given by:

\[
\nu = \frac{k_{\text{cat}} [E] [S]}{K_m + [S]} \tag{2.5}
\]

or

\[
\nu = \frac{V_{\text{max}} [S]}{K_m + [S]} \tag{2.6}
\]

where, \( K_m = \text{Michaelis constant} = \frac{(k_{-1} + k_{\text{cat}})}{k_1} \)

Comparing the Briggs-Haldane equation (equation 2.6) with the Henri-Michaelis-Menten equation (equation 2.4), we find that the main difference is the substitution of \( K_m \) for \( K_S \). The Michaelis constant, \( K_m \), is numerically equivalent to the substrate concentration that yields half-maximal velocity and is usually measured in molar units. The value of \( K_m \) usually ranges from \( 10^{-6} \) to \( 10^{-2} \) M. When \( k_{\text{cat}} \ll k_{-1} \), \( K_m \equiv k_1 / k_{-1} \equiv K_S \). In other words, when \( k_{\text{cat}} \ll k_{-1} \), \( K_m \) is essentially the dissociation constant, and the Briggs-Haldane equation reduces to the Henri-Michaelis-Menten equation. However, when \( k_{-1} \ll k_{\text{cat}} \), \( K_m \equiv k_{\text{cat}} / k_1 \); that is, \( K_m \) is a kinetic
constant. Although $K_m$ is not always equivalent to $K_S$, it is generally used as a relative measure of the substrate binding affinity of the enzyme. A lower $K_m$ indicates higher substrate affinity. The value of $K_m$ is a constant for a given enzyme and is, hence, used to compare enzymes from different sources.

Equation (2.6) may be rearranged to give $(V_{\text{max}} - v)(K_m + [S]) = K_m V_{\text{max}}$ or $(a-y)(b+x) = \text{constant}$. This equation represents a right rectangular hyperbola. Thus a plot of velocity versus substrate concentration of an enzyme-catalyzed reaction that follows Henri-Michaelis-Menten or Briggs-Haldane kinetics gives a hyperbolic curve.

At $[S] \ll K_m$ equation (2.5) reduces to,

$$v = \frac{k_{\text{cat}} [E][S]}{K_m}$$

(2.7)

The ratio $k_{\text{cat}}/K_m$ is the second order rate constant for the enzymatic reaction and is commonly used to compare the substrate specificity and catalytic efficiency of enzymes. In comparing different substrates for an enzyme, the largest differences are often found in the value of $k_{\text{cat}}$ rather than $K_m$. This is because $K_m$ represents the substrate binding in the ground state while substrate specificity depends on binding of the substrate in its transition state. Thus, the ratio $k_{\text{cat}}/K_m$ provides a better estimation of productive substrate binding and hence, the catalytic efficiency and substrate specificity of an enzyme.

The above equations are for the cases where a single substrate molecule is converted to a single product molecule. In other words, it is a uni-uni reaction. In cases where two substrate molecules react to give one product molecule, the rate equation becomes more complicated by the fact that binding of one substrate could affect the affinity of the enzyme for the second substrate molecule (Fig. 2-3, pg. 39). In such cases the rate equation is given by equation 2.8.
Fig. 2-3 Schematic representation of a bi-uni reaction in which binding of one substrate increases the affinity for the second substrate by a factor $\alpha$.

$$\left(\frac{v}{V_{\text{max}}} = \frac{[A]}{\alpha K_A \left(1 + \frac{K_B}{[B]}\right) + [A] \left(1 + \frac{\alpha K_B}{[B]}\right)} \right)$$

where $A$ and $B$ are the two substrate molecules with intrinsic dissociation constants $K_A$ and $K_B$, respectively, and $\alpha$ is the factor by which binding of one substrate molecule changes the dissociation constant for the other substrate molecule. When $\alpha = 1$, binding of $A$ has no effect on the affinity of the enzyme for $B$. When $\alpha < 1$, binding of $A$ increases the affinity of the enzyme for $B$.

2.3 Enzyme Inhibition

A molecule that reversibly reduces the rate of an enzyme-catalyzed reaction is called an inhibitor. It is usually a molecule that mimics the substrate, product, or transition state formed in the normal reaction. On the basis of the effect of the inhibitor on the kinetic constants of the enzyme, three types of inhibitors may be defined.
(i) Competitive inhibitors

A competitive inhibitor is a molecule that binds to the enzyme reversibly and prevents it from binding the substrate. Thus a competitive inhibitor and the substrate are mutually exclusive (Fig. 2-4, pg. 40).

\[
E + S \overset{K_S}{\underset{K_i}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P + I
\]

Fig. 2-4 Schematic representation of competitive inhibition

In the presence of a competitive inhibitor, the enzyme appears to have a lower affinity for the substrate due to the competition between the substrate and the inhibitor molecule for the same active site. In other words, a competitive inhibitor affects the apparent value of \(K_m\) of the substrate. Since the ES complex still dissociates into product at the same rate, \(V_{max}\) remains unaffected. The rate equation for an enzyme-catalyzed reaction in the presence of an inhibitor of concentration [I] will be given by equation (2.9).

\[
v = \frac{V_{max} [S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (2.9)
\]

\(K_i\) is the dissociation constant for the inhibitor “I”. The degree of inhibition caused by a competitive inhibitor depends on [S], [I], \(K_m\) and \(K_i\). The lower the value of \(K_i\), the greater is the
degree of inhibition at any given \([I]\) and \([S]\). Since the substrate and the inhibitor compete for the same site on the enzyme, an increase in \([S]\) at a constant \([I]\) decreases the degree of competitive inhibition, while an increase in \([I]\) at a constant \([S]\) increases the degree of competitive inhibition.

(ii) Non-competitive inhibition

A non-competitive inhibitor binds reversibly to a site separate from the substrate binding site. It can bind to \(E\) and \(ES\), although the \(ESI\) complex is inactive. Thus, a pure non-competitive inhibitor affects the \(V_{\text{max}}\) of the enzyme catalyzed reaction leaving the \(K_m\) unaffected (Fig. 2-5, pg. 41).

\[
\begin{align*}
\text{E} + \text{S} & \rightleftharpoons_{K_S} \text{ES} \\
& \overset{k_{\text{cat}}}{\longrightarrow} \text{E} + \text{P}
\end{align*}
\]

\[
\begin{align*}
\text{E} + \text{I} & \rightleftharpoons_{K_I} \text{EI} \\
& \overset{K_I}{\longrightarrow} \text{ES} \\
\text{E} + \text{S} & \rightleftharpoons_{K_S} \text{ESI}
\end{align*}
\]

\(\text{Fig. 2-5 Schematic representation of non-competitive inhibition}\)

The rate equation for an enzyme catalyzed reaction shown in Fig. 2-5 (pg. 41) will be given by equation (2.10).
\[
\frac{V_{\text{max}}}{1 + \frac{[I]}{K_i}} \quad [S]
\]

where

\[
\frac{V_{\text{max}}}{1 + \frac{[I]}{K_i}} = \text{apparent } V'_{\text{max}} \text{ at a given } [I]
\]

Unlike the competitive inhibition where the degree of inhibition varies with [S], the degree of non-competitive inhibition depends only on [I] and \(K_i\).

(iii) **Uncompetitive inhibition**

An uncompetitive inhibitor binds to only the ES complex, not to the free enzyme, and the ESI complex is non-productive (Fig. 2-6, pg. 42).

\[
\begin{align*}
E + S & \overset{K_S}{\rightarrow} ES \\
ES & \overset{k_{\text{cat}}}{\rightarrow} E + P \\
+ & \\
I & \\
\downarrow & \\
K_i & \\
\rightarrow ESI
\end{align*}
\]

Fig. 2-6 Schematic representation of uncompetitive inhibition
Thus an uncompetitive inhibitor affects both $V_{\text{max}}$ and $K_m$. The rate equation for the system shown in Fig. 2-6 (pg. 42) is given by equation (2.11).

$$v = \frac{V_{\text{max}}}{\left(1 + \frac{[I]}{K_i}\right)} \frac{[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]}$$

(2.11)

The three types of inhibition can be easily distinguished from an inverse plot of rate ($v$) versus substrate concentration, $[S]$ (Fig. 2-7, pg. 43).

![Fig. 2-7 The 1/v versus 1/[S] plots for the three types of inhibition.](image-url)
2.4 Experimental Procedures

2.4.1 Materials and General Procedures.

(i) Materials

The bacterial strains DH5α and Y1090 were obtained from Gibco/BRL and Stratagene, respectively. The plasmid pRE1 which was used for the overexpression of wild-type L-Ru5P 4-epimerase and for all mutagenesis purposes was prepared by Dr. Martin E. Tanner. The plasmid pTAB01 used for the overexpression of ribulokinase was subcloned in our lab by Dr. Paul Morgan. The chemicals for preparing the LB media and the anion exchange resin DE52 were from Difco Laboratories. Restriction enzymes EcoRI and PstI were from Promega. The dNTP mix was from Gibco/BRL. Protease inhibitors pepstatin and aprotinin were from Boehringer Mannheim. The PWO polymerase used in all mutagenesis were from Roche Diagnostics. The antibiotic ampicillin was from Fisher Biotech. All protein dialysis was done using Spectra/Por dialysis tubing (MWCO 12 – 14 kDa) from Spectrum Laboratories Inc. The disposable cuvettes used for kinetic assays were from Sarstedt.

All PCR primers used for mutagenesis and sequencing were synthesized at the Nucleic Acid and Protein Services (NAPS) unit at the University of British Columbia. The recombinant genes obtained from site-directed mutagenesis were also sequenced at the NAPS unit.

The chemicals used for synthesis and all coupling enzymes were from Sigma-Aldrich.
(ii) General Procedures

Purification of plasmids was done using the Wizard™ Minipreps Plasmid DNA Purification system from Promega. The concentrations ($\chi$) of double-stranded plasmids were determined from their absorbance at 260 nm using the following formula:

$$\chi \; (\mu g/mL) = A_{260} \times 50$$

The concentrations of primers were also determined from absorbance at 260 nm using the following formula:

$$\chi \; (\text{picomole}/\mu l) = A_{260} \times \frac{100}{1.5N_A + 0.7N_C + 1.2N_G + 0.84N_T}$$

where $N_A$, $N_C$, $N_G$, and $N_T$ are the number of adenosine, cytidine, guanosine and thymidine bases in the primer.

Protein concentrations were determined from the absorbance at 280 nm ($\varepsilon = 1.73 \text{ mL/mg/cm}$).63

One unit of enzyme is defined as the amount of enzyme required to convert 1 μmole of substrate into product in 1 min.

Unless otherwise stated, proteins were always handled at 4 °C and stored at -80 °C in buffers containing 10% glycerol. The general techniques in molecular biology were those described in Short Protocols in Molecular Biology.95
2.4.2 Site-Directed Mutagenesis

Plasmid pRE1 (3.75 kb),\(^\text{63}\) an expression vector that encodes for the \textit{araD} gene (Appendix-3), was used as a template for all mutagenesis experiments. The D76E mutant was prepared by the method of recombinant circle polymerase chain reaction (RCPCR) (Fig. 2-8, pg.

Fig. 2-8 Steps involved in site-directed mutagenesis by recombinant circle polymerase chain reaction (RCPCR)
Primers used for preparing this mutant, with the bases altered for mutation and restriction site underlined are given in Table 2-1 (pg. 49). The double stranded template plasmid, pRE1, was first incubated at 37 °C for 1 hr with 2 units of the restriction enzyme EcoRI and PstI in two separate reactions. This produced two linearized plasmids. Primers JS01 and MT09 were used to amplify the plasmid cut with EcoRI and primers MT08 and JS02 were used to amplify the plasmid cut with PstI. Each PCR reaction mixture contained 5 μL of 10 x concentrated polymerase buffer, 5 μL of 2.5 mM dNTP mix, 50 picomole of each primer, 20 ng of pRE1 template plasmid, and 2.5 units of Pwo polymerase in a total volume of 50 μL. The reaction mixture was topped with mineral oil and amplification was done using the following cycles: one cycle of 2 min at 96 °C, 10 cycles of 1 min at 96 °C, 45 sec at 60 °C and 2 min at 72 °C, 10 cycles of 1 min at 96 °C, 45 sec at 55 °C and 2 min at 72 °C, 10 cycles of 1 min at 96 °C, 45 sec at 50 °C and 2 min at 72 °C, followed by one cycle of 7 min at 72 °C. In order to generate recombinant circles, equal volume aliquots of the two crude PCR products were combined and underwent the following temperature transformations: 3 min at 94 °C, 2 hrs at 50 °C. After returning to room temperature, the recombinant circles were transformed into E. coli DH5α to generate the final plasmid pJSTB1 encoding the D76E mutation (please see subsequent paragraph for details of cell growth and plasmid purification).
Fig. 2-9 Steps involved in the QuickChange™ site-directed mutagenesis protocol

All other mutants were prepared according to the protocol of the QuickChange™ Site-Directed Mutagenesis kit from Stratagene (Fig. 2-9, pg. 48). Complementary oligonucleotide pairs that were used to introduce the mutations are listed in Table 2-1 (pg. 49) with the altered nucleotides underlined. The PCR (Polymerase Chain Reaction) reaction mixture for amplification of the plasmid contained 5 µL of 10 x concentrated polymerase buffer, 5 µL of 2.5 mM dNTP mix, 50 picomole of each primer, 20 ng of pRE1 template plasmid, and 2.5 units of PWO polymerase in a total volume of 50 µL. The reaction mixture was topped with 30 µL of mineral oil to prevent evaporation and passed through the following cycles in an MJ Research minicycler: one cycle of 1 min at 96 °C, 10 cycles of 30 sec at 96 °C, 1 min at 60 °C and 8 min at
68 °C, followed by 10 cycles of 30 sec at 96 °C, 1 min at 55 °C and 8 min at 68 °C followed by one cycle of 7 min at 72 °C. The PCR product was then treated with 10 units of DpnI restriction enzyme to digest the template plasmid, and transformed immediately into DH5α cells and plated on an LB agar plate containing 100 μg/mL ampicillin. Single colonies from the plate were used to induce 10 mL LB media containing 100 μg/mL ampicillin. The cells were allowed to grow overnight at 37 °C to an OD<sub>600</sub> of 1.8 and harvested by centrifugation at 5000 rpm for 5 min. The plasmids were isolated and purified using the Wizard™ Minipreps Plasmid DNA Purification system and submitted to NAPS unit for sequencing. The sequences were manually checked for the introduction of the correct mutation and the presence of any undesired mutation.

Table 2-1. Primers used for site-directed mutagenesis and sequencing of L-Ru5P 4-epimerase. The bases altered for the desired mutation have been underlined.

<table>
<thead>
<tr>
<th>Primers for D76E mutation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JS01</strong>: 5' - GCC CTC CTC CGA GAC CCC AAC TCA CC -3'</td>
<td></td>
</tr>
<tr>
<td><strong>MT09</strong>: 5' - GCA GAG CGA GGT ATG TAG GCG GTG - 3'</td>
<td></td>
</tr>
<tr>
<td><strong>JS02</strong>: 5' - GGT GAG TTG GGG TCT CGG AGG AGG GC -3'</td>
<td></td>
</tr>
<tr>
<td><strong>MT08</strong>: 5' - CGA CGA GCG TGA CAC CAC GAT GCC -3'</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primers for T116Y mutation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JS07</strong>: 5' - CCA GCA ACC GGC TAC ACC CAC GCC GAC T -3'</td>
<td></td>
</tr>
<tr>
<td><strong>JS08</strong>: 5' - AGT CGGGCTTGG TGT AGC CGG TTG CTG G -3'</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primers for T116E mutation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JS09</strong>: 5' - CCA GCA ACC GGC <strong>GAG</strong> ACC CAC GCC GAC T -3'</td>
<td></td>
</tr>
<tr>
<td><strong>JS10</strong>: 5' - AGT CGG CGT GGG TCT <strong>CGC</strong> CGG TTG CTG G -3'</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1 (contd.)

Primers for E142Q mutation

JS11: 5'-CAA CGG CGA ATA TCA GTG GGA AAC CG -3'
JS12: 5'-CCG GTT TCC CAC TGA TAT TCG CCG TTG -3'

Primers for H218N mutation

JS13: 5'-CGC TGC TGG ATA AAA ACT ATC TGC GTA AGC -3'
JS14: 5'-GCC ATG CTT ACG CAG ATA GGT TTT ATC CAG CAG -3'

Primers for D120N mutation

JS15: 5'-GGC ACC ACC CAC GCC AAC TAT TTC TAC GGC -3'
JS16: 5'-GGT GCC GTA GAA ATA GTT GGC GTG GG -3'

Primers for N28A mutation

JS17: 5'-CTC ACA TGG GGC GCC GTC ATG TGC TAC GGC G -3'
JS18: 5'-CGC TGA CGG CGC CCC ATG TGC AGC GCC G -3'

Primers for K42M mutation

JS19: 5'-GCG TCT TTG TGA TCA TGC CTT CCG GCC TCG -3'
JS20: 5'-GCC GGA AGG CAT GAT CAC AAA GAC GCC G -3'

Primers for sequencing

JS05: 5'-CGA AAC CGG TGA AGT GGT TG -3'
JS06: 5'-GCC GTT GAT TTC TGC GTC GG -3'

2.4.3 Overexpression and Purification of Wild-Type and Mutant Epimerases

The WT and mutant epimerases were purified by a process similar to that reported by Johnson and Tanner. For the WT epimerase, the plasmid pRE1 was transformed into CaCl2 competent E. coli Y1090, a strain lacking a functional epimerase gene (Stratagene). Individual
colonies grown on LB (Luria Bertani) agar plates containing 100 μg/mL ampicillin were used to innoculate 4 x 500 mL LB medium containing 100 μg/mL ampicillin. The cells were allowed to grow overnight to an OD₆₀₀ of 1.8 – 2.0, harvested by centrifugation and then resuspended in 50 mL 10 mM potassium phosphate buffer (pH 7.0, containing 10% glycerol, 1 μg/mL pepstatin and 1 μg/mL aprotinin). The cells were lysed using a pre-cooled French pressure cell at 20,000 psi and the cell debris was removed by centrifugation at 8,000 rpm for 30 min. The supernatant containing the epimerase was partially purified by ammonium sulfate precipitation at 40% saturation. The precipitate was collected by centrifugation at 8,000 rpm for 20 min, resuspended in buffer-A (10 mM potassium phosphate buffer, pH 7.0, containing 10% glycerol) and dialyzed twice against 600 mL fresh buffer-A using a 12-14 kDa MWCO dialysis tubing. The partially purified protein was then loaded on a 100 mL anion exchange DE-52 cellulose column pre-equilibrated with buffer-A at room temperature. The column was first washed with buffer-A and then eluted with 1 liter of 0 to 0.4 M NaCl linear gradient in buffer-A. Fractions containing the epimerase were identified by SDS-PAGE, pooled, concentrated using centrifugal filters (Amicon), dialyzed and then applied to a 5 mL Hi Trap Q Sepharose HP anion exchange column (Pharmacia) that had been pre-equilibrated with buffer B (10 mM potassium phosphate buffer, pH 7.6, containing 10% glycerol) at room temperature. The epimerase was eluted with 0 to 0.4 M NaCl linear gradient in buffer B. Fractions containing the purified protein were pooled, concentrated and stored at -80 °C. For the mutant epimerases, the respective plasmids were transformed into CaCl₂ competent E. coli Y1090 and purified by a procedure similar to that used for the WT epimerase.
2.4.4 Preparation of Zinc Substituted Enzymes

The WT and mutant apoenzymes (enzymes stripped of metal) were prepared by incubating the enzyme with 20 equiv. EDTA for 6 hrs. The excess EDTA was removed by extensive dialysis against metal free HEPES-Tris buffer (50 mM, pH 7.6 containing 10% glycerol). The apoenzyme was then reconstituted with 4 equiv. 99.99% pure ZnCl₂ and dialyzed once against metal-free HEPES-Tris buffer. For the H97N mutant, the apoenzyme was reconstituted with 2 equiv. of ZnCl₂ without dialysis. The metal free buffer was prepared by the method given in the literature.

2.4.5 Crystallization of D120N Mutant

The purified D120N mutant was first washed with deionized H₂O to remove the phosphate buffer and then concentrated to ~10 mg/mL using centrifugal filters (Ultrafree centricons, Millipore). It was crystallized using the hanging drop vapor diffusion method. The crystals used for data collection were obtained using a precipitant similar to that previously reported. The drops were set up with an equal volume of ~10 mg/mL protein sample in the purification buffer and a precipitant composed of 4.0 M sodium formate and 0.1% n-octyl-β-D-glucoside. The square-shaped crystals grew to a typical size of 0.5 mm x 0.5 mm x 0.2 mm in a week.

The diffraction data was collected by Yu Luo (a Post-doctoral fellow with Dr. Natalie Strynadka, at the Department of Biochemistry, University of British Columbia) at the Brookhaven National Laboratory beamline 12C using a Brandeis-4 CCD detector system. The structure was determined by the molecular replacement method using the wild-type L-Ru5P 4-epimerase as the model.
2.4.6 Synthesis of L-Ru5P

Fig. 2-10 Synthesis of L-Ru5P

Crude L-ribulose was prepared by the procedure of Anderson (Fig. 2-10, pg. 53). L-Arabinose (25 g) was refluxed in 200 mL dry pyridine for 4 hrs. The pyridine was evaporated down to about 50 mL and the unreacted L-arabinose was removed by precipitation with ethanol. The filtrate was evaporated to give crude L-ribulose (2.5 g) as a brown oil that was used in the enzymatic synthesis of L-Ru5P without further purification.

The enzyme L-ribulokinase used for the synthesis was overexpressed by transforming the pTAB01 plasmid into the *E. coli* strain Y1090 that lacks a functional epimerase. A single colony from a LB agar plate containing 100 μg/mL ampicillin was used to inoculate 500 mL LB medium containing 100 μg/mL ampicillin. The cells were grown at 37 °C to an OD$_{600}$ of 0.6-0.8, induced with 5 mM IPTG and then allowed to grow to an OD$_{600}$ of 1.5-2.0. The cells were harvested by centrifugation at 5000 rpm for 20 min and then resuspended in 1 mM glutathione (reduced) buffer, pH 7.0, containing 1 mM EDTA. The cells were lysed using a pre-cooled French pressure cell at 20,000 psi and the cell debris was removed by centrifugation at 8000 rpm for 30 min. The crude cell lysate was directly used for synthesis.
Crude L-ribulose (0.6 g, 4 mmol) was phosphorylated using half of the ribulokinase preparation and ATP according to the procedure of Anderson, and the resulting L-Ru5P was purified as described previously. Concentrations of L-Ru5P solutions were determined enzymatically using the epimerization assay conditions in the presence of an excess of L-Ru5P 4-epimerase (0.15 units/mL).

2.4.7 Synthesis of Glycolohydroxamate

Benzyloxyacetyl chloride (3 g, 16.2 mmol), benzyloxyamine (4 g, 32.4 mmol), and triethylamine (18.5 mL) were refluxed in 150 mL of CH$_2$Cl$_2$ for 4 hrs under an argon atmosphere. The solution was washed with 1 N HCl and saturated sodium bicarbonate and then evaporated to dryness under reduced pressure. Purification by silica gel chromatography (3:7 EtOAc/hexane) gave 2.2 g (50%) of dibenzylated glycolohydroxamate as a white solid: $^1$H NMR (CDCl$_3$) $\delta$ 8.75-8.85 (br s, 1 H), 7.2-7.4 (m, 10 H), 4.9 (s, 2 H), 4.5 (s, 2 H), 4.1 (s, 2 H); LC-MS 272 (M+H$^+$). The dibenzylated glycolohydroxamate (2.2 g, 8.1 mmol) was dissolved in 10 mL of EtOAc, and 0.22 g of 10% Pd/C was added. The mixture was stirred under 1 atm of hydrogen at room temperature for 10 hrs and then filtered. The filtrate was evaporated to dryness under reduced pressure to give 700 mg (95%) of glycolohydroxamate as a light yellow oil: $^1$H NMR (D$_2$O) $\delta$ 4.1 (s, 2 H); DCI-MS 92 (M+H$^+$).
2.4.8 Epimerase Assay

The epimerization reaction in the L-Ru5P to D-Xu5P direction was assayed at 37 °C by a method similar to that reported in literature (Fig. 2-15, pg. 63). The assay mixture contained 25 mM Gly-Gly buffer (pH 7.6), 0.125 mM ZnCl\(_2\), 5 mM ribose-5-phosphate, 0.1 mM TPP, 0.15 mM NADH, 0.25 units transketolase, 2.5 units α-glycerophosphate dehydrogenase (αGDH), 25 units triosephosphate isomerase, 0.005 units L-Ru5P 4-epimerase and varying amounts of L-Ru5P. The assay mixture was pre-incubated for 10 min prior to initiation to allow an impurity of an alternate substrate in the L-Ru5P (approximately 3%) to be consumed by the coupling enzymes. The reaction was initiated by the addition of the epimerase and followed by the decrease in absorbance of NADH at 340 nm (ε\(_{340}\) = 6.22 mM\(^{-1}\) cm\(^{-1}\)). The initial reaction velocities were fitted into equation 2.6 using the program Grafit to obtain the kinetic constants.

2.4.9 Assay for Aldolase Activity

Both stopped assay and continuous assay was used to measure the activity of L-Ru5P 4-epimerase catalyzed condensation reaction between 1,3-dihydroxyacetone (DHA) and glycolaldehyde phosphate (GAP). The Ca\(^{2+}\)-salt of glycolaldehyde phosphate was prepared by Anne Johnson of our lab according to the method of Müller \textit{et al}. The Ca\(^{2+}\)-salt of glycolaldehyde phosphate (60 mg) in 20 mL H\(_2\)O was stirred with the Na\(^{+}\)-form of Amberlite IR-120 (plus) resin (the H\(^+\) form was converted to the Na\(^{+}\) form by repeated washing with 10% NaCl followed by washes with H\(_2\)O until the pH was neutral) until it had completely dissolved. The resin was filtered off and the filtrate was lyophilized. The solid Na\(^{+}\) form of glycolaldehyde phosphate was stored at −20 °C.
For the stopped assay, 0.5 mg L-Ru5P 4-epimerase was incubated with 5 mM glycolaldehyde phosphate and 50 mM dihydroxyacetone phosphate at 37 °C. At timed intervals a 50 μL aliquot of the reaction mixture was taken out and added to a cuvette containing 25 mM Gly-Gly buffer (pH 7.6), 0.125 mM ZnCl₂, 5 mM ribose 5-phosphate, 0.1 mM TPP, 0.15 mM NADH, 0.25 units transketolase, 2.5 units α-glycerophosphate dehydrogenase (αGDH), 25 units triosephosphate isomerase and 0.15 units of WT epimerase at 37 °C. The total pentose phosphates formed was determined by following the decrease in absorbance at 340 nm (consumption of NADH).

The specific activity of the WT and mutant epimerases for the aldol condensation reaction was determined using a continuous assay similar to that used for measuring the epimerization reaction. However, in this case, instead of L-Ru5P, 5 mM GAP and 50 mM DHA were added as the substrate (Fig. 2-18, pg. 70). Large amounts of L-Ru5P 4-epimerase (0.3 - 0.5 mg) had to be added to see a measurable rate. GAP has been found to be an alternate substrate for α-GDH. At concentrations above 5 mM GAP, the background rate becomes quite significant. Hence, all kinetic measurements were performed at concentrations of GAP below 5 mM. Each cuvette containing the assay mixture was first allowed to equilibrate at 37 °C for 5 min. The background rate due to GAP was measured in the first 5 min of the reaction and then the aldolase reaction was initiated by adding DHA. The background rate was subtracted from the final steady state rate after initiation to obtain the rate of the aldolase reaction.

To obtain the kinetic constants for the aldolase reaction in the WT and H97N mutant, the initial rates for varying concentrations of GAP were determined at five different fixed concentrations of DHA. Reciprocal initial velocities were plotted against the reciprocal of the variable substrate concentration to obtain intersecting initial velocity patterns.
2.5 Results and Discussion

2.5.1 Mutagenesis of Active-Site Residues

L-Ru5P 4-epimerase and L-Fu1P aldolase share 26% sequence homology and the three-dimensional structures of the two enzymes show a very close similarity (over 192 C$_\alpha$ pairs, RMSD = 1.5 Å) (Fig. 2-2, pg. 32). Despite the fact that the enzymes share a common fold and catalyze mechanistically similar reactions, the structures of the substrates demand that there be significant differences in the residues involved in binding and/or catalysis. In both reactions, the metal serves as an electrophilic catalyst and must be coordinated to the ketone oxygen at C-2 to promote enolate formation (Fig. 2-1, pg. 30). In addition, both enzymes must position catalytic acid/base residues appropriately to deprotonate a hydroxyl group at C-4 of the respective substrates (two such residues are presumably required for the epimerase, one for each epimer). However, the phosphate of L-Fu1P is located at C-1, whereas that of L-Ru5P is at C-5. Since the phosphate is likely a key determinant in the recognition and binding of these substrates, it is reasonable to assume that the enzymes would evolve a distinct phosphate-binding pocket. This is certainly the case with the aldolase, and it is important to note that the residues in the phosphate-binding pocket of the aldolase are strictly conserved in the epimerase. If these residues play a similar role in the epimerase, the substrate would have to bind in a reverse orientation, and the acid/base residue(s) corresponding to those in the aldolase would be mis-positioned to participate in catalysis.
Fig. 2-12 Structure of substrates for L-Ru5P 4-epimerase and L-FulP aldolase

Fig. 2-13 Active site of L-Ru5P 4-epimerase (green and red) superimposed on that of L-Fu1P aldolase complexed with inhibitor (yellow). The red and green colored residues represent two adjacent subunits in the epimerase. Shown in black is the aldolase inhibitor phosphoglycolohydroxamate. Residues in the epimerase have been labeled.
Three groups of residues in the active site of the epimerase were targeted for site-directed mutagenesis studies (Fig. 2-13, pg. 58). The first were those that aligned with the putative acid/base catalysts of the aldolase in structure and sequence comparison. As mentioned earlier, the WT epimerase shows residual aldolase activity. It was thought that by reintroducing the aldolase side chains back into the epimerase, one might dramatically increase the background aldolase activity. At the time the work was initiated, reports on the aldolase-phosphoglycolohydroxamate complex structure implicated Glu73 and Tyr113' (Tyr113' indicates that the Tyr113 is coming from the subunit adjacent to the one bearing the active site zinc) as the residues important for acid/base catalysis in L-Fu1P aldolase. The corresponding residues in the epimerase are Asp76 and Thr116'. Both these residues are completely conserved among the epimerases. The mutations D76E and T116Y were aimed at converting these residues back to the corresponding aldolase residues in order to increase the background aldolase activity. An additional mutant T116E was made in an attempt to introduce a potential acid/base residue into the position occupied by Thr116.

The second group of mutants was chosen in an attempt to identify the phosphate binding pocket of the epimerase. Five conserved residues were implicated as serving this role due to both their sequence homology and their structural similarity with the corresponding residues in the aldolase. These include, Asn28, Ser44, Gly45, Ser74, and Ser75 (Fig. 2-13, pg. 58). One of these conserved residues, Asn28 was mutated to alanine. Since asparagine likely binds to the phosphate group via a H-bond, converting it into an alanine should eliminate this interaction. The crystal structure of the epimerase shows that Lys42 is located in close proximity to this conserved pocket. This lysine residue has been found to be fully conserved among the epimerases, but is absent among the aldolases. The role of Lys42 in substrate binding was investigated by changing it into a methionine residue, which is structurally similar to lysine but
does not bear the positive charge that could potentially bind the negative charge on the phosphate group.

The final group of mutants focused on residues that could play the role of acid/base catalysts and serve to deprotonate the hydroxyls of the epimeric substrates in the two reaction directions (Fig. 2-1, pg. 30). Previous studies have shown that Asp76, which is homologous to Glu73, the acid/base catalyst in the aldolase, is not a key catalytic residue in the epimerase. A survey of all potential active-site acid/base catalytic residues in the epimerase structure led to the selection of three candidates for mutagenesis- Asp120’, His218’ and Glu142 (Fig. 2-13, pg. 58). The aspartate and the histidine were mutated to asparagine, while the glutamate was mutated to glutamine. These mutations were designed to remove the ability of the residues to act as acid/base catalysts while preserving the steric size and shape as much as possible.

The mutations were introduced into the template plasmid pRE1 using PCR-based mutagenesis techniques. While the D76E mutant was prepared by the RCPCR method all other mutants were prepared by the QuickChange™ mutagenesis (QSDM) kit from Stratagene. The RCPCR method involves linearization of the plasmid using two restriction enzymes, amplification of the two linearized plasmids, recombination and then transformation into E. coli (Fig. 2-8, pg. 46). The QSDM method is based on the fact that the template plasmid is dam methylated (i.e., the adenosine residue in the sequence GATC is methylated) and hence can be digested with the restriction enzyme DpnI (Fig. 2-9, pg. 48). This allows mutagenesis to be achieved without linearization of the plasmid and hence is a much more efficient and rapid process. The plasmids obtained from mutagenesis were sequenced and checked for introduction of any unwanted errors. The plasmids encoding the mutations D76E, T116Y, T116E, E142Q, H218N, D120N, N28A, and K42M have been named pJSTB1, pJSTB2, pJSTB3, pJSTB4,
pJSTB5, pJSTB6, pJSTB7, and pJSTB8, respectively. The plasmid for the H97N mutant was prepared by Dr. Anne E. Johnson in our lab.

2.5.2 Overexpression, Purification and Characterization of WT and Mutant Epimerases

The wild-type and mutant epimerases were overexpressed by transforming the respective plasmids into the *E. coli* strain Y1090 which lack a functional epimerase. (Please refer to Appendix-2 for a definition of plasmid). The bacterial strain *E. coli* Y1090 was used to avoid contamination of the mutant enzymes with the wild-type epimerase originating from the bacterial chromosomal DNA. All nine mutants, D120N, H218N, E142Q, K42M, N28A, D76E, T116E, T116Y and H97N were expressed and purified to >90% homogeneity as determined by SDS-PAGE (Fig. 2-14, pg. 61). With the exception of T116E and T116Y, all the mutants
behaved similar to the wild-type epimerase during the purification procedure. The T116Y and T116E mutants exhibited a greater tendency to bind to the anion exchange column.

It has been shown previously that the overexpressed enzyme grown in culture contains a mixture of the following metals as listed in order of decreasing abundance: \( \text{Zn}^{2+} > \text{Mn}^{2+} > \text{Cu}^{2+} \). Furthermore, the nature of the metal bound to the enzyme affects its catalytic ability. In order to ensure homogeneity, all purified enzymes were stripped of their metals using EDTA and reconstituted with \( \text{Zn}^{2+} \). Consistent with earlier reports, it was observed that the H97N mutant tended to lose its metal during purification and dialysis. Because of this, the H97N apoenzyme was reconstituted with 2 equivalents of \( \text{ZnCl}_2 \) and was not dialyzed after reconstitution. The molecular weights of the mutant enzymes, as determined by ESI-MS, were consistent with that expected from their amino acid sequences. The WT and mutants epimerases were further characterized by circular dichroism (CD) in the range 190 – 240 nm. All mutants except for T116Y and T116E showed identical CD spectra to that of the wild-type enzyme, suggesting the secondary structure was largely unchanged by the mutation (data not shown). In the case of the T116Y and T116E mutants, the CD spectra showed a greatly reduced ellipticity at 208 and 222 nm suggesting that these mutants were not properly folded.
2.5.3 Kinetic Analysis of the Mutant Epimerases

![Chemical structures and reactions]

**Fig. 2-15 Coupled assay for L-Ru5P 4-epimerase**

The wild-type and mutant L-Ru5P 4-epimerases were assayed for epimerase activity in the L-Ru5P to D-Xu5P direction in the presence of 0.125 mM Zn$^{2+}$ using the coupled assay shown in Fig. 2-15 (pg. 63). The kinetic constants obtained for the wild-type and mutant epimerase are summarized in Table 2-2 (pg. 64).
Table 2-2. Kinetic constants for L-Ru5P with WT and mutant epimerases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (sec$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (mM$^{-1}$ sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>19.4 ± 0.4</td>
<td>0.047 ± 0.006</td>
<td>4.14 x 10$^2$</td>
</tr>
<tr>
<td>D76E</td>
<td>1.0 ± 0.03</td>
<td>0.25 ± 0.02</td>
<td>4.0</td>
</tr>
<tr>
<td>K42M</td>
<td>-</td>
<td>-</td>
<td>0.032</td>
</tr>
<tr>
<td>N28A</td>
<td>2.5 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>2.1</td>
</tr>
<tr>
<td>D120N</td>
<td>0.0057 ± 0.0002</td>
<td>0.28 ± 0.02</td>
<td>0.020</td>
</tr>
<tr>
<td>H218N</td>
<td>0.81 ± 0.02</td>
<td>0.58 ± 0.04</td>
<td>1.4</td>
</tr>
<tr>
<td>E142Q</td>
<td>2.1 ± 0.02</td>
<td>0.086 ± 0.002</td>
<td>25.0</td>
</tr>
</tbody>
</table>

The errors reported here are the standard deviations of the points that make the rate versus [substrate] curve. Each experiment was repeated three times and the results were found to be reproducible within a 10% error margin.

Of the three epimerase mutants prepared in an attempt to increase the background aldolase activity, only the D76E mutant could be characterized kinetically. The other two mutants, T116Y and T116E, did not show any significant activity and were likely improperly folded as suggested by their CD spectra. The decrease in the value of $k_{\text{cat}}$ for the D76E mutant was not significant enough to ascribe any direct role in catalysis for Asp76. However, it has been suggested that Asp76 is coordinated to the Zn$^{2+}$ ion through a water molecule. A mutation of this residue to a bulkier Glu76 could have disrupted this second sphere of coordination resulting in a decrease in the value of $k_{\text{cat}}$. The 20-fold increase in the value of $K_m$ observed with this mutant could be due to the introduction of a larger residue in the active site.
The Asn28 and Lys42 residues were targeted for mutagenesis studies to test whether the phosphate binding pocket in the aldolase is conserved in the epimerase. The results obtained with N28A and K42M support the notion that both enzymes utilize the same phosphate-binding pocket (Table 2-2, pg. 64). Asn28 is a strictly conserved residue in the epimerase and its counterpart, Asn29 in the aldolase is also strictly conserved. In the aldolase, Asn29 forms a hydrogen bond with the phosphate of phosphoglycolohydroxamate in the aldolase-inhibitor complex. The N28A mutant shows a 27-fold increase in the value of $K_m$, while the $k_{cat}$ value showed only an 8-fold decrease as compared to the wild-type enzyme. This is consistent with the proposal that Asn28 plays an important role in substrate binding in the epimerase. Lys42 is located just below the phosphate-binding pocket of the epimerase; however, no counterpart is found at the comparable position in the aldolase. The K42M mutant could not be saturated even up to 2.5 mM L-Ru5P suggesting that the value of $K_m$ is extremely high. This indicates that substrate binding has been impaired and is consistent with the idea that the positive charge of Lys42 is important for recognizing the negatively charged phosphate. It should be noted that the $K_m$ value of the wild-type epimerase (0.047 mM) is significantly lower than that of the aldolase (2.2 mM). This higher substrate affinity in the wild-type epimerase as compared to the wild-type aldolase could be due to the presence of Lys42.

Considering the fact that the substrates for the epimerase and the aldolase are phosphorylated at opposite ends and the phosphate binding pocket is conserved between the two enzymes, it follows that the substrates would be oriented in opposite directions in the two enzymes. Starting from L-Ru5P in the epimerase and L-Fu5P in the aldolase, both substrates need to be deprotonated at the C-4 hydroxyl. If the substrates are oriented in opposite directions in the two enzymes, it follows that the residue homologous to the catalytic residue in the aldolase, namely Asp76, will not be correctly positioned to act as the catalytic residue in the
epimerase. Previous work done in our lab has shown that a mutation of Asp76 to Asn did not considerably affect the catalytic efficiency of the enzyme. We mutated Asp76 to Glu and did not see any significant decrease in the value of $k_{\text{cat}}$ suggesting that this residue is not one of the catalytic acid/base residues in L-Ru5P 4-epimerase.

Fig. 2-16 A). A single crystal of the D120N mutant. B). The superposition of the wild-type and D120N active site. The zinc, carbon, oxygen and nitrogen atoms are colored in gold, green, red and blue, respectively. For the D120N mutant all atoms are colored in beet.

On the basis of crystal structure data, we had identified three alternative candidates as potential catalytic residues - Asp120', His218' and Glu142. Both Asp120' and His218' extend into the active site (represented by the position of Zn$^{2+}$) from the adjacent subunit (hence they are represented as Asp120' and His218'), whereas Glu142 is from the same subunit. The residues Glu142, His218' and Asp120' are conserved among the epimerases but not among the aldolases. The D120N mutant shows a 3000-fold decrease in the value of $k_{\text{cat}}$ while the value of $K_m$ remains almost unchanged. This strongly supports the notion that Asp120' is one of the
catalytic residues involved in deprotonating the C-4 hydroxyl of one of the substrates. In order to confirm that the dramatic decrease in the activity of the D120N mutant was not due to a structural perturbation, this mutant was crystallized. The X-ray diffraction data was solved by Dr. Yu Luo in the lab of Dr. Natalie Strynadka by the molecular replacement method. The superposition of the crystal structure of the wild-type epimerase and the D120N mutant clearly shows that the mutation did not perturb the structure of the enzyme (Fig. 2-16, pg. 66).

Fig. 2-17 Ribbon views of the substrate-binding sites of L-Ru5P 4-epimerase with bound substrates (A) L-Ru5P and (B) D-Xu5P based on molecular models of the complexes. The secondary structural elements from two adjacent monomers that form the active site are shown in gray and magenta, respectively. Active site residues are shown in a ball-and-stick representation with green carbon, red oxygen, and blue nitrogen atoms. Residues that protrude from the second monomer are labeled with a prime symbol. The substrates are colored with cyan carbon, red oxygen, and magenta phosphate atoms.
The mutants H218N and E142Q displayed $k_{\text{cat}}$ values that were reduced from that of the wild-type by only 24-fold and 9-fold, respectively, and their $K_m$ values were comparable to that of the wild-type epimerase. The values of the kinetic constants with these mutants indicate that they do not play key roles in substrate binding or catalysis. The crystal structure of the epimerase shows that His218' is directly H-bonded to Asp120' and Thr116'. The 24-fold reduction in the value of $k_{\text{cat}}$ in H218N mutant could be due to a loss in its ability to assist the Asp120' residue in catalysis.

Sometime towards the end of this work, Cleland et al. reported that a mutation of Tyr229' to phenylalanine lowers the value of $k_{\text{cat}}$ by about 1000-fold, while the binding constant is not greatly affected. Tyr229' likely extends into the active site from the adjacent subunit but could not be located in the X-ray crystal structure since it resides among the 8 residues of the disordered C-terminal tail. On the basis of both our work and Cleland’s, we propose that Asp120' and Tyr229' are the two acid/base catalytic residues involved in the protonation/deprotonation of the C-4 hydroxyl. Substrate docking studies with the WT epimerase (done by Dr. Yu Luo, Dept. of Biochemistry, UBC) suggests that Tyr229' is responsible for deprotonating L-Ru5P and Asp120' is responsible for deprotonating D-Xu5P (Fig. 2-17, pg. 67). Asp120' and Tyr229' are completely conserved among the epimerases, while in the aldolases these residues are either absent or replaced by an aliphatic residue. Although the last 8 residues of the C-terminus (224 – 231) were not visible they have been proposed to form a flap that closes over the active site of the adjacent subunit. It is possible that when the substrate binds, the C-terminus flap closes and presents Tyr229', catalysis occurs, and then the flap opens to release the product. In the crystal structure of the aldolase, the corresponding flap region (C-terminal residues 207 to 215) is also missing. However, an aldolase mutant in which the flap region has been deleted has a $k_{\text{cat}}$ value that is reduced by only
15-fold and a $K_m$ value that is increased by almost 50 fold suggesting that in the aldolase, the C-terminus flap region is probably important for binding the substrate but does not play a major role in catalysis.\textsuperscript{62}

\subsection*{2.5.4 Aldolase Activity of WT and Mutant Epimerases}

The wild-type and the mutant L-Ru5P 4-epimerases were also studied for their ability to catalyze the aldol condensation between dihydroxyacetone (DHA) and glycolaldehyde phosphate (GAP). In past work, the aldolase activity has been measured using a stopped assay that followed the rate of production of the epimeric pentose phosphates (L-Ru5P and D-Xu5P) from 50 mM dihydroxyacetone and 5 mM glycolaldehyde phosphate.\textsuperscript{63, 64} This assay suffered from the fact that the pentose phosphate products bound much more tightly than DHA or GAP and were therefore potent competitive inhibitors. To avoid this problem, the aldolase activity was measured using a continuous spectrophotometric assay for D-Xu5P that was a modification of the normal epimerase assay in which the substrate L-Ru5P was replaced with dihydroxyacetone and glycolaldehyde phosphate.

One key to making this assay work was the realization that glycolaldehyde phosphate is a slow, alternate substrate for one of the coupling enzymes, $\alpha$-glycerophosphate dehydrogenase ($\alpha$-GDH) (the value of $K_m$ for GAP was determined to be about 15 mM), and therefore, leads to a background rate of NADH oxidation. For this reason, the amount of $\alpha$-GDH in both the epimerase and aldolase activity assays was reduced from the 5 units/mL\textsuperscript{99} used in the original assay to 2.5 units/mL. By keeping the concentration of glycolaldehyde phosphate at or below 5 mM, it was possible to keep the background rate at acceptable levels and still maintain coupled conditions. A drawback to this assay is that it only detects the production of D-Xu5P whereas
the epimerase is presumably forming both epimers in its background aldol reaction. In the case of the wild-type enzyme and most of the mutants, the epimerase activity is still much greater than the background aldolase activity and therefore any L-Ru5P that is formed will readily be epimerized and detected by this assay. In assays of mutants that have extremely low levels of epimerase activity (e.g. D120N), however, the observed rate may reflect the rate of formation of only D-Xu5P, as opposed to that of total pentose phosphate. Despite these limitations, the assay does not suffer from competitive inhibition since the products are continuously removed by the action of the coupling enzymes.

Fig. 2-18 Continuous assay for measuring the aldolase activity in L-Ru5P 4-epimerase
Attempts to fully delineate the kinetics of the background aldolase activity of the wild-type enzyme using the coupled assay were unsuccessful due to an extremely high value of $K_m$ for dihydroxyacetone (>150 mM). It was evident, however, that at saturating levels of glyceraldehyde phosphate (5 mM) the value of $aK_m$ (equation 2.8) for dihydroxyacetone decreased to about 50 mM.

Table 2-3. Aldolase activity with 5 mM glycolaldehyde phosphate and 50 mM dihydroxyacetone

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Aldolase specific activity ($\mu$mol min$^{-1}$ mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$8.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>D76E</td>
<td>$1.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>D120N</td>
<td>$1.2 \times 10^{-3}$</td>
</tr>
<tr>
<td>H218N</td>
<td>$0.55 \times 10^{-3}$</td>
</tr>
<tr>
<td>E142Q</td>
<td>$12.2 \times 10^{-3}$</td>
</tr>
<tr>
<td>K42M*</td>
<td>$1.4 \times 10^{-3}$</td>
</tr>
<tr>
<td>N28A</td>
<td>$6.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>H97N</td>
<td>$6.3 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

* Excess zinc was found to inhibit the aldolase activity for this mutant, so the enzyme was used directly as purified and Zn$^{2+}$ was omitted from the assay.

- Results reproducible within 10% error.

The observed aldolase activities for the WT and mutant epimerases using 50 mM dihydroxyacetone and 5 mM glycolaldehyde phosphate are shown in Table 2-3 (pg. 71). The H97N mutant had previously been studied using the stopped assay in our lab by Dr. Anne
Johnson. It was reported that this mutant catalyzed the aldol condensation reaction at a rate faster than the wild-type epimerase. In subsequent work, Cleland and co-workers also reported that H97N showed aldolase activity but the rate was similar to that of the wild-type enzyme. In order to clarify the conflicting reports we studied the aldolase activity of this mutant with a continuous assay. A direct measure of the specific activity clearly shows that the rate of aldol condensation of GAP and DHA by the H97N mutant is comparable to that of the wild-type epimerase. It is likely that the initial report of increased aldolase activity by the H97N mutant was mistaken since the aldol reaction run in the direction of pentose phosphate formation is extremely sensitive to product inhibition. Since this is more severe in the case of the wild-type enzyme, the H97N mutant seemed to catalyze the aldol reaction faster at later time points.

Initially, three mutants were targeted in an attempt to increase the residual background aldolase activity; these were D76E, T116Y and T116E. Initial reports had suggested that Glu73 and Tyr113' were the two catalytic residues in the aldolase. In the epimerase, these residues are conserved as Asp76 and Thr116'. In the crystal structure, Asp76 of the epimerase aligns with Glu73 of the aldolase (Fig. 2-13, pg. 58). It was thought that increasing the length of the side chain in the epimerase by one methylene group may permit this residue to protonate the metal-bound enolate and generate the aldol products. Similar logic led to the investigation of T116E and T116Y. The resulting kinetic values for both epimerase (Table 2-2, pg. 64) and aldolase activities (Table 2-3, pg. 71) of the D76E mutant, however, were similar to those obtained for the wild-type enzyme. It is likely that the glutamate side chain of this mutant remained deprotonated throughout the epimerase reaction or was mis-positioned to participate in catalysis since the orientation of the bound substrate had changed. The two mutants of Thr116' lacked any detectable epimerase or aldolase activity. It is likely that
the increased steric bulk introduced by the mutations caused a conformational change that was incompatible with catalysis. This notion was supported by observed differences between the CD spectra of these mutants and the wild-type enzyme (data not shown) and by inspection of the X-ray structure that shows Thr116' is 100% buried at the tetrameric interface by contacts with Tyr141, His97, and His171 of the neighboring monomer. Towards the end of this work, Joerger et al. showed that Tyr113' is not a catalytic residue in L-Fu1P aldolase.\textsuperscript{62} Instead, Glu73 acts as the catalytic residue for both deprotonating the C-4 hydroxyl and protonating the enolate intermediate. In light of this recent report, no further studies were performed with Thr116' epimerase mutants.

The low levels of residual aldolase activity displayed by the epimerase served as one of the first pieces of evidence supporting the proposed retroaldol/aldol mechanism for this enzyme.\textsuperscript{63} It is quite puzzling, therefore, to see that most of the mutants displayed a background aldolase activity similar to that of the wild-type enzyme (0.0085 μmol min\textsuperscript{-1} mg\textsuperscript{-1}). The largest difference was a 15-fold decrease in activity for H218N. Mutants of the phosphate-binding pocket (K42M and N28A) as well as that of the catalytic residue (D120N) all showed aldolase activity that was within 10-fold of that observed with the wild-type enzyme (Table 2-3, pg. 71). Cleland and co-workers also observed that the residual aldolase activity with the Y229F mutant (mutant of one of the catalytic residues) was comparable to that of the wild-type enzyme.\textsuperscript{64} One possible explanation could be that binding/release of the intermediates is the rate-determining step in this very slow reaction for both the mutants and the wild-type enzyme. Since the enzyme has evolved to avoid intermediate release, this barrier may be much higher than the barriers to catalysis even with the catalytically impaired mutants. A similar argument can be made if deprotonation/protonation of the dihydroxyacetone/enolate was the rate-determining step since this event is not a part of the normal catalytic process. At this point, it is unclear whether the
deprotonation of dihydroxyacetone occurs in solution or in the enzyme active site. An alternate explanation is that the epimerase utilizes different catalytic residues and a different phosphate-binding pocket to promote the aldol addition of dihydroxyacetone to glycolaldehyde phosphate. This would be the case if the reaction does not occur in the active site; however, this seems unlikely given that the addition is slowed by severe product inhibition. This could also happen if the aldolase activity arose from the binding of the pentose sugars/glycolaldehyde phosphate in an orientation that differs from the one it assumes during the epimerization reaction. An inspection of the active site reveals a potential alternate phosphate-binding pocket supplied by residues Arg221, Lys217, and Lys222. In this scenario, the altered orientation of binding could allow an acid/base residue to protonate the enolate and release the aldol products. During epimerization, however, the intermediates would be formed in the correct orientation and an acid/base residue would not be able to protonate the enolate.

### 2.5.5 Inhibition Studies

![Diagram of inhibitors](image)

Fig. 2-19 A comparison of the structure of intermediates and inhibitors of (A) L-Ru5P 4-epimerase (potential) and (B) L-Fu1P aldolase.
Molecules that resemble the substrate or the intermediate inhibit the enzyme by binding to the active site and preventing the substrate from binding. Such molecules are called competitive inhibitors (please refer to section 2.3, pg. 40 for a description of competitive inhibitors). Since enzymes have evolved to bind high energy intermediates and transition states tighter than the ground state of the substrate, intermediate and transition state analogues are known to be good competitive inhibitors. The reactions catalyzed by both the aldolase and the epimerase proceed via an enolate intermediate and hydroxamates have been shown to resemble enolate intermediates in several cases.\textsuperscript{100}

Phosphoglycolohydroxamate is known to be a potent inhibitor of L-Fuc1P aldolase ($K_i = 3.1$ $\mu$M), as it mimics the enolate of dihydroxyacetone phosphate (Fig. 2-19, pg. 74).\textsuperscript{57, 59} Co-crystallization of this inhibitor with the aldolase led to a detailed picture of how the reaction intermediate likely binds to the aldolase. In the case of L-Ru5P 4-epimerase, the corresponding inhibitor would be glycolohydroxamate since it should mimic the enolate of dihydroxyacetone. Glycolohydroxamate was prepared in 40% overall yield by the condensation of benzyloxyacetyl chloride and benzyloxyamine followed by deprotection with catalytic hydrogenation. To use the coupled assay for inhibition studies, it was first necessary to determine if the hydroxamate interfered with the action of the coupling enzymes, particularly triosephosphate isomerase, which is known to be inhibited by phosphoglycolohydroxamate.\textsuperscript{101} When D-Xu5P (1.1 mM) was used as a substrate in the coupled assay (Fig. 2-15, pg. 63), 45% inhibition was observed with 5 mM glycolohydroxamate. However, the $K_m$ value of the epimerase for L-Ru5P is 0.04 mM, and if the hydroxamate is a good competitive inhibitor of the epimerase ($K_i \leq 0.1$ mM), we should be able to see inhibition at a concentration of 0.1 mM. At this concentration of glycolohydroxamate, inhibitory effects on the coupled assay were negligible. Studies on the
inhibition of L-Ru5P epimerase were then performed keeping the hydroxamate concentration at 0.1 mM, and no significant inhibition could be detected. To check if synergistic binding was important, the study was repeated with 0.1 mM glycolohydroxamate and 1 mM glycolaldehyde phosphate; however, no synergistic inhibition could be detected.

2.6 Conclusions

L-Ru5P has been shown to catalyze the interconversion of L-Ru5P and D-Xu5P via a retroaldol/aldol mechanism. It shares 26% sequence identity and 40% sequence similarity with L-Fu1P aldolase. Both L-Ru5P 4-epimerase and L-Fu1P aldolase require a divalent metal ion for catalysis and the metal binding ligands are conserved between the two enzymes. The crystal structures of L-Ru5P 4-epimerase and L-Fu1P aldolase show considerable similarity. On the basis of the crystal structure of L-Fu1P aldolase, Glu73 and Tyr113' were initially suggested to be the two catalytic residues in that enzyme. The Tyr113' residue extends into the active site from the adjacent monomer unit. However, in a more recent report, Schultz et al. have suggested that Glu73 is the only catalytic residue and that Tyr113' is involved in proper positioning of the substrate. The residue corresponding to Glu73 in the aldolase is Asp76 in the epimerase, and previous work as well as results in this thesis has shown that Asp76 is not a catalytic residue in L-Ru5P 4-epimerase.

On the basis of sequence homology and examination of the crystal structure of the epimerase, site-directed mutagenesis studies were designed to determine the catalytic residues and the nature of substrate binding in the epimerase. The K42M and N28A mutants were prepared to study the phosphate binding pocket of the epimerase. A mutation of the Asn28 residue to an alanine increased the value of $K_m$ by about 30 fold, while the $k_{cat}$ value remained almost unchanged. This shows that the substrates utilize the same phosphate binding pocket in
both the epimerase and the aldolase. The other conserved residues in the phosphate-binding pocket of the epimerase are Ser74, Ser75, Ser44 and Gly45. However, in addition to these residues, Lys42 was also seen near this pocket in the crystal structure. The positive charge on lysine could play an important role in binding the negative charge of the phosphate group. The mutation of this residue to methionine resulted in a dramatic increase in the value of $K_m$, supporting the role of Lys42 in substrate binding. The aldolase has a $K_m$ value of 2.2 mM for L-Fu1P and lacks the corresponding lysine. The epimerase has a $K_m$ value of 0.04 mM for L-Ru5P and this difference might be attributed to a better binding of the phosphate group in the presence of a positively charged lysine.

Considering the fact that the substrates of L-Ru5P 4-epimerase and L-Fu1P aldolase are phosphorylated at opposite ends and the phosphate binding pocket is conserved between the two enzymes, it is not surprising that the catalytic residues are different in the epimerase and the aldolase. Three residues – Glu142, His218' and Asp120' were investigated as potential catalytic residues. Kinetic studies show that Asp120' is an excellent candidate for one of the catalytic residues involved in deprotonating the C-4 hydroxyl of one of the substrates. Cleland and co-workers have shown that Tyr229' is another critical catalytic residue. By docking L-Ru5P and D-Xu5P into the active site of the WT epimerase, it has been proposed that Tyr229' is responsible for deprotonating L-Ru5P and that Asp120' is responsible for deprotonating D-Xu5P (Fig. 2-17, pg. 67). Thus, while there is a single residue, Glu73, responsible for both deprotonating L-Fu1P and reprotonating the enolate intermediate, there are likely two separate residues in L-Ru5P 4-epimerase. It is interesting to note that both the catalytic residues in L-Ru5P 4-epimerase come from the subunit adjacent to the one bearing the active site zinc.
Attempts to convert the epimerase back into an aldolase by a single mutation were not successful. The key to aldolase activity is the protonation of the enolate intermediate. In L-Fu1P aldolase, Glu73 has been proposed to be the catalytic residue involved in the protonation of the enediolate intermediate. The corresponding residue in the epimerase is Asp76; however, conversion of Asp76 into a glutamate did not improve the aldolase activity of the epimerase. In fact, the mutation seems to have lowered the specific activity by about a factor of 7. In light of the fact that the substrates of the epimerase and the aldolase bind in “flipped” orientations, this may not be surprising. Asp76 may no longer be in the correct position to protonate the enolate. It would be quite interesting to test whether the wild-type epimerase or the D76E mutant could catalyze the L-Fu1P aldolase reaction itself (conversion of L-Fu1P into dihydroxyacetone phosphate and L-lactaldehyde or vice versa). L-Fu1P should bind in the correct orientation for catalysis to occur.

Previous work on this enzyme has shown that the epimerization reaction proceeds via a retroaldol/aldol mechanism involving a dihydroxyacetone enolate intermediate. The hydroxamate analogues of the enolate intermediate are known to be good inhibitors, as they mimic the enolate structurally and show a higher affinity for metals. Glycolohydroxamate was designed to mimic the dihydroxyacetone enediolate intermediate (Fig. 2-19, pg.74). However, no inhibition of the WT or H97N mutant was observed with glycolohydroxamate in the presence or absence of GAP. This lack of inhibition of the epimerization reaction by the hydroxamate could be suggestive of the inaccessibility of the active site in conformation that normally binds the intermediate or the possibility that the enolate is very loosely bound to the metal.
From the available structural and mechanistic studies, it is clear that the epimerase and the aldolase have evolved from a common ancestor and both have retained the ability to use a divalent cation in promoting carbon-carbon bond cleavage/formation reactions via a retroaldol/aldol process. They have also retained the use of a conserved phosphate-binding pocket that is required for substrate recognition. The different positioning of the phosphate in their respective substrates, however, dictates that the substrates must bind in a reversed or "flipped" orientation. For this reason, the enzymes have recruited completely different residues to serve as acids and bases during catalysis. We have also been able to identify the catalytic residues involved in this reaction. The mutagenesis studies done on the epimerase also indicates that in spite of the high structural and sequence similarity between L-Ru5P 4-epimerase and L-Fu1P aldolase, the substrate bound active site configuration is quite different in the two enzymes. A better understanding of the substrate bound active site configuration may be reached by studying the crystal structure of the substrate bound enzyme. Surprisingly all mutants showed aldolase activity comparable to that of the WT epimerase including the two mutants of the catalytic residues. One explanation for the activity in the latter cases could be that D120N could still produce L-Ru5P and Y229F could still produce D-Xu5P. An attempt was therefore made to determine which one of the two epimers is formed by the aldolase activity of D120N. However, since the residual epimerase activity in the D120N mutant was still appreciable as compared to the aldolase activity, the first-formed product of aldol condensation could not be determined. It would be interesting to prepare a double mutant D120N/Y229F and see the effect on the aldolase activity. This would help in determining whether the amino acid residues involved in catalyzing the aldolase reaction in the epimerase are the same or different from the amino acid residues involved in catalyzing the epimerization reaction. It would also be interesting to do mutagenesis studies on the alternate phosphate binding pocket containing
Arg221, Ly217 and Ly222. If a mutation in one of these residues results in an enzyme lacking the aldolase activity, but retaining the epimerase activity, it would suggest that the residual aldolase activity in L-Ru5P 4-epimerase arises from binding of the pentose sugar phosphate in a different orientation within the active site. Once we have this information it would be easier to do site-directed mutagenesis to convert the epimerase into an aldolase.
3 UDP-\textit{N}-Acetylglucosamine 2-Epimerase

UDP-\textit{N}-acetylglucosamine 2-epimerase (UDPE) is a homodimeric allosteric enzyme that catalyzes the interconversion of UDP-GlcNAc and UDP-ManNAc. Previous work with this enzyme shows that it catalyzes the reaction through a C-O bond cleavage/formation mechanism (Fig. 3-1, pg. 81). In the UDP-GlcNAc to UDP-ManNAc direction the \textit{anti}-elimination of UDP with deprotonation at C-2 gives enzyme-bound glycal and UDP as intermediates. A subsequent \textit{syn}-addition of UDP with protonation at C-2 from the opposite face gives the epimer, UDP-ManNAc. Evidence for this mechanism comes from the observation of intermediate release, positional isotope exchange (PIX) and structural homology between UDPE and glycosyltransferases (discussed in detail in Chapter 1, Section 1.3.2.2b, Pg. 19).

Questions that remain to be answered with this enzyme include the nature of catalytic residues involved in deprotonating/reprotonating C-2 and the nature of allosteric regulation. Here we have used site-directed mutagenesis to identify one of the potential catalytic residues of

\begin{center}
\includegraphics[width=\textwidth]{figure3-1.png}
\end{center}

\textit{Fig. 3-1 Proposed mechanism for the reaction catalyzed by UDP-GlcNAc 2-epimerase}
UDPE. The roles of some other conserved active-site residues involved in substrate binding and cooperativity have also been investigated. We have used a combination of site-directed mutagenesis, kinetics and binding studies to gain a better understanding of the cooperativity in UDPE.

3.1 Allostery

Allostery involves the activation or inhibition of an enzyme by non-covalent binding of a molecule(s) that may not necessarily bear any structural similarity to the substrate(s) or product(s) of the reaction catalyzed by that enzyme. The molecule that causes the activation or inhibition is called the modulator or effector and the enzyme that shows the phenomenon of cooperativity is called an allosteric enzyme. The modulator ligand binds non-covalently to a "regulatory site" which is usually separate from the "active site". When the modulator and the substrate molecule are the same, the phenomenon is called homotropic cooperativity and when the modulator and the substrate molecules are different it is called heterotropic cooperativity. Homotropic cooperativity is usually activating (positive) while heterotropic cooperativity may be activating (positive) or inhibitory (negative).
Glycogen phosphorylase, which catalyzes the formation of glucose 1-phosphate from glycogen and inorganic phosphate (Fig. 3-2, pg. 83) is an example of an enzyme exhibiting heterotropic cooperativity.\(^{102}\) It is a homodimer that exists in two forms: phosphorylase \(a\) and phosphorylase \(b\). The less active form of the enzyme, phosphorylase \(b\), is converted into the active form, phosphorylase \(a\), by phosphorylation of serine-14 by the enzyme phosphorylase kinase (Fig. 3-3, pg. 84). Another enzyme, phosphorylase phosphatase, converts the active form into the inactive form by hydrolysis of the same phosphate group. AMP is an allosteric activator of phosphorylase \(b\) while ATP, glucose, glucose 6-phosphate and caffeine are its allosteric inhibitors. AMP has no effect on the activity of phosphorylase \(a\). Phosphorylase \(a\), however, is allosterically inhibited by glucose and caffeine. The structures of both glycogen phosphorylase \(a\) and glycogen phosphorylase \(b\) have been solved and it has been found that there is a single regulatory site for the modulators AMP, ATP, glucose and glucose 6-phosphate.\(^{103}\)
Fig. 3-3 Covalent and allosteric regulation in glycogen phosphorylase

An example of homotropic cooperativity is found in yeast hexokinase which converts glucose into glucose 6-phosphate using MgATP$^2-$. (Fig. 3-2, pg. 83). It shows allosteric activation by phosphate, citrate, 3-phosphoglycerate and its own substrate MgATP$^2-$.\textsuperscript{104} In solution, the monomeric and dimeric forms of the enzymes exist in equilibrium. In the absence of ligands, the enzyme is virtually all in the dimeric form. It has been observed that dissociation of the dimer is enhanced by the addition of glucose and MgADP.\textsuperscript{105} However, the presence of glucose and MgATP$^2-$ shifts the equilibrium towards the dimer. Furthermore, the initial velocity of the dimeric hexokinase is about 10 times higher than the monomeric hexokinase.\textsuperscript{106} The structure of the monomeric and dimeric yeast hexokinase complexed with sugar and nucleotide substrates shows a separate allosteric binding site for the nucleotide at the dimer interface.\textsuperscript{107} It has been proposed that MgATP$^2-$ activates the enzyme by binding to the dimer interface and stabilizing the more active form of the enzyme.
3.2 Molecular Models of Cooperativity

In multimeric non-regulatory enzymes, subunits behave independently. In other words, the substrate binding and catalytic activity in one subunit has no effect on the substrate binding and catalytic activity in other subunits. The rate of the reaction catalyzed by such enzymes is defined by the Henri-Michaelis-Menten equation (eqn. 2.6) and the rate ($v$) versus substrate concentration, [S], curve is a rectangular hyperbola with a fixed curve (Fig. 3-4, pg. 86). The ratio of substrate concentrations required to bring about a fixed percentage change in rate is constant for such enzymes. Thus, an 81-fold increase in substrate concentration is required for an increase in rate from 10% to 90% of $V_{\text{max}}$. There are several examples of such enzymes, one of them being L-ribulose-5-phosphate 4-epimerase, discussed in detail in Chapter 2.

$$v = \frac{V_{\text{max}} [S]}{K_m + [S]}$$

(2.6)

In allosteric enzymes, however, non-covalent ligand binding at one site affects the substrate binding and/or catalysis at other sites through long-range conformational changes within the enzyme. It is this cooperativity between binding sites and/or subunits that is responsible for the characteristic sigmoidal rate curve. Enzymes showing cooperativity are much more sensitive to changes in the concentration of substrates and/or modulators. In a dimeric enzyme that shows allosteric regulation by its substrate, the rate could increase from 10% to 90% of $V_{\text{max}}$ with a minimum of a 9-fold increase in substrate concentration as opposed to an 81-fold increase in non-cooperative enzymes. A tetrameric allosteric enzyme could bring about the same change in rate with a minimum of a 3-fold increase in substrate concentration (Fig. 3-4, pg. 86). In general, the greater the cooperativity, the lower the ratio of substrate required to bring about a change in rate from 10% to 90% $V_{\text{max}}$. Thus cooperativity provides a
simple mechanism for increased sensitivity towards changes in concentration of metabolites within a cell.

Fig. 3-4 A comparison of rate curves for two homo-tetrameric enzymes that have the same $V_{\text{max}}$. (A) Hyperbolic response. (B) Sigmoidal response.

There are two models that try to explain allosteric regulation. One is the "symmetry model" or "MWC model" proposed by Monod, Wyman and Changeux and the other is the "sequential model" or "KNF model" proposed by Koshland, Némethy and Filmer.

(a) Symmetry model or MWC model

The basic assumptions of the MWC model are as follows: 108

(i) Allosteric proteins are oligomers in which the subunits are associated in such a way that the enzyme molecule possesses at least one axis of symmetry.
(ii) For every ligand that can bind to the enzyme there exists one, and only one, binding site per subunit. In other words, the symmetry of each set of binding sites is the same as the symmetry of the molecule.

(iii) The enzyme can exist in two interconvertible conformations, T (tense) and R (relaxed), which are in equilibrium. The two conformations differ in their quaternary structure and/or strength of bonds between the subunits.

(iv) Transitions from one state to the other are concerted, i.e., the configurations of all subunits change simultaneously so that the symmetry of the enzyme is conserved.

(v) The T and the R conformations differ in their affinity for allosteric ligands. Thus the equilibrium between the two forms is shifted by the presence of the modulator although the binding sites do not interact with each other.

A.

\[
\begin{align*}
T_0 & \overset{K_T}{\longrightarrow} R_0 \\
T_0 + L & \overset{K_T}{\longrightarrow} T_1 \\
T_1 + L & \overset{K_T}{\longrightarrow} T_2 \\
& \vdots \\
T_{n-1} + L & \overset{K_T}{\longrightarrow} T_n \\
R_0 + L & \overset{K_R}{\longrightarrow} R_1 \\
R_1 + L & \overset{K_R}{\longrightarrow} R_2 \\
& \vdots \\
R_{n-1} + L & \overset{K_R}{\longrightarrow} R_n
\end{align*}
\]

B.

\[
\begin{align*}
T_0 & \overset{K_T}{\longrightarrow} R_0 \\
R_0 & \overset{K_R}{\longrightarrow} R_1 \\
R_1 & \overset{K_R}{\longrightarrow} R_2
\end{align*}
\]

Fig. 3-5 Schematic representation of MWC model
A schematic representation of the MWC model is shown in Fig. 3-5A (pg. 87).\textsuperscript{93,109} The free enzyme exists in equilibrium between the T and R forms and the equilibrium constant for the interchange of one state to the other is $K_t$.

$$K_t = \frac{[T_0]}{[R_0]}$$

where $T_0$ and $R_0$ are the enzymes in the T and R forms, respectively, with no bound ligand molecules. Since $K_t$ is large in the absence of any modulator ligand, the free enzyme exists largely in the T-state. Each binding site of the enzyme in the R state can bind the ligand molecule with an intrinsic dissociation $K_R$ no matter how many ligand molecules are already bound. Similarly, each binding site of the enzyme in the T form can bind the ligand molecule with an intrinsic dissociation constant of $K_T$.

Thus, in the MWC model, binding of a ligand at one site does not alter its affinity at other sites, but the two forms of the enzyme differ in their affinities for the same ligand. In the case of an enzyme showing homotropic allostery, the substrate binds preferentially to the high affinity R-state shifting the $T_0 - R_0$ equilibrium to the right (Fig. 3-5, pg. 87). Thus, more high affinity sites are available to subsequent substrate molecules resulting in a sharp rise in the rate curve at low substrate concentrations. As the substrate concentration is increased, eventually all enzyme molecules will exist in the high affinity R-form. Close to saturation, there will be fewer binding sites available to the added substrate molecules and the rate curve flattens out to give a sigmoidal shape. In its simplest form, the MWC model assumes that the substrate binds exclusively to the R-state. Such a situation is shown in Fig. 3-5B (pg. 87).
(b) Concerted model or KNF model

The basic assumptions of the KNF model are as follows: 110

(i) Each subunit in the oligomeric enzyme can exist in two (or more) different conformational states.

(ii) The conformation of a subunit depends on the number and nature of ligands bound.

(iii) Change from one conformation to the other occurs as a result of ligand binding.

(iv) The conformational change in one subunit upon ligand binding may be transmitted to neighboring vacant subunits through inter-subunit interactions.

The KNF model thus allows for two (or more) conformations for each subunit. Unlike the MWC model which requires that all subunits in an oligomer be in the same conformation, the KNF model allows for hybrid oligomers containing subunits with different conformations. Another difference is that in the MWC model the conformational change happens before ligand binding, whereas in the KNF model the conformational change could happen before, after or simultaneously with ligand binding.

In a simple scenario, if A and B are the two possible conformations for a subunit, $K_t$ is the transformation constant for conversion of A to B and $K_S$ is the intrinsic affinity of the subunit for ligand L, then the conversion of an unoccupied subunit to an occupied subunit may occur in the following ways: 93
In all the above cases $K_t K_s$ is the intrinsic binding constant of a vacant site irrespective of the sequence of events. The KNF model also allows interaction between subunits or binding sites which affect the binding constant of a site. In a dimeric enzyme, if $K_{AA}$, $K_{AB}$ and $K_{BB}$ are the interaction constants for AA pair, AB pair and BB pair, respectively, the ligand binding events may be shown as follows:

$$K_t K_s = K'_t K'_s = K''_t K''_s$$

For the purpose of simplicity, $K_{AA}$ is assumed to be 1 and $K_{AB}$ and $K_{BB}$ are represented as stability of AB and BB pairs relative to AA pair.

$$K_{AB} = \frac{[AB][A]}{[AA][B]} \quad \text{and} \quad K_{BB} = \frac{[BB][A][A]}{[AA][B][B]}$$

A $K_{BB}$ value > 1 indicates that complexes with BB interactions are more stable than complexes with AA interactions. It is the relative values of $K_{AB}$ and $K_{BB}$ that determine whether
the cooperativity is positive or negative. Thus, when $K_{AB} \geq 1$ and $K_{BB} > K_{AB}$, the binding constants of vacant sites increase as sites are occupied resulting in positive cooperativity. On the other hand, when $K_{AB} > 1$ but $K_{BB} < 1$, then the binding constants of vacant sites decrease as sites are occupied resulting in negative cooperativity.

(c) General model

Fig. 3-7 Schematic representation of a general model for allostery. "L" represents a ligand molecule. The 1\textsuperscript{st} and the 3\textsuperscript{rd} column (dotted lined box) represents the MWC model and the diagonal (solid lined box) represents the KNF model.

A general model that is intermediary between the sequential and concerted models is usually believed to be true for most allosteric enzymes (Fig. 3-7, pg. 91).\textsuperscript{93} The KNF model allows greater flexibility in the conformational changes induced by the ligand molecule. By allowing for interactions between subunits the KNF model is able to explain negative cooperativity, which cannot be explained by the MWC model. Moreover, the KNF model allows for situations in which a conformational change upon substrate binding does not
necessarily result in cooperativity. This is observed in many oligomeric enzymes that are non-cooperative. The flexibility and ability of the KNF model to explain both positive and negative cooperativity make it a more realistic model. On the other hand the advantage of MWC model is its simplicity. Experimentally it is hard to prove one model or the other.

3.3 K-systems and V-systems

In the models discussed above it was assumed that the activator ligand (in the case of homotropic cooperativity, it’s the substrate) binds preferentially to the R-form (MWC model) or B-form (KNF model) and stabilizes it. Such systems in which, (i) the T- and R-forms of the enzyme have same catalytic efficiency but differ in their affinities (MWC model) or (ii) binding of a ligand molecule alters the binding constant ($K_S$) of vacant sites (KNF model) leaving $V_{max}$ unaffected are called K-systems. The sigmoidicity of the rate curve in a K-type homotropic allosteric enzyme results from a greater number of high affinity sites available for subsequent substrate molecules. Thus, factors that lower the cooperativity between the binding sites result in an increase in the value of $K_m$, app. An inhibitor of a K-system preferentially binds to and stabilizes the T-form. Thus, it behaves like a competitive inhibitor and lowers the affinity of the enzyme for the substrate without affecting the $V_{max}$. Consequently, as we increase the inhibitor concentration, the sigmoidicity of the [substrate] versus rate curve increases.

Systems in which the T- and R-states have the same affinity for the substrate but differ in their catalytic efficiency are known as V-systems. In a V-type homotropic allosteric enzyme the binding of the substrate to one site increases the catalytic efficiency of the vacant sites. Thus the subsequent substrate molecules will have more high efficiency sites available, resulting in a sigmoidal rate curve. Factors that lowers the allostery in the enzyme would result in a decrease in the value of $V_{max}$, app. An inhibitor of a V-system behaves in a non-competitive manner,
lowering the $V_{\text{max}}$ while leaving the $K_{m,\text{app}}$ unaffected. If the T-state has no catalytic activity, the inhibitor will be purely non-competitive. However, if both T and R states are catalytically active, then the activator will be non-essential and the inhibitor will be partially non-competitive.

The $K$- and $V$-systems are further discussed below in section 3.4.

### 3.4 Kinetics in Allosteric Enzymes

Fig. 3-8 Scheme for a reaction catalyzed by a dimeric allosteric enzyme (K-system). E, S and P are the free enzyme, substrate and product, respectively. ES, SE and SES are the various enzyme-substrate complexes.

Consider a homodimeric enzyme that shows homotropic cooperativity with respect to substrate binding (K-system) (Fig. 3-8, pg. 93). Each subunit of the enzyme has one substrate binding site with an intrinsic dissociation constant, $K_S$. Due to cooperativity between binding sites binding of one substrate molecule increases its affinity at the second vacant site by a factor $a$. Thus, the dissociation constant for binding of substrate to the enzyme-substrate complex (SE
or ES) is $aK_s$. Taking into account the distribution of enzyme between E, ES, SE and SES, the rate equation is given by:

$$\frac{v}{V_{\text{max}}} = \frac{[S]}{K_s} + \frac{[S]^2}{aK_s^2}$$

(3.1)

$$1 + \frac{2[S]}{K_s} + \frac{[S]^2}{aK_s^2}$$

However, due to cooperativity between substrate binding sites, at any point in time there will be a higher concentration of enzymes existing as ES$_2$ (or SES) complex as compared to ES or SE complex. In fact, if the cooperativity is high enough, the concentration of ES would be negligible at any [S] that is appreciable as compared to $K_s$. Under such conditions the above equation will reduce to:

$$\frac{v}{V_{\text{max}}} = \frac{[S]^2}{aK_s^2}$$

(3.2)

$$1 + \frac{[S]^2}{aK_s^2}$$

or

$$\frac{v}{V_{\text{max}}} = \frac{[S]^2}{aK_s^2 + [S]^2}$$

(3.3)

or

$$\frac{v}{V_{\text{max}}} = \frac{[S]^2}{K_{m, \text{app}} + [S]^2}$$

(3.4)
where \( K_{m, app} = aK_S^2 \) \( (3.5) \)

In general, for an enzyme with \( n \) equivalent substrate binding sites with high cooperativity (i.e., the interaction factors \( a, b, c, \) etc. are very small numbers), the concentration of all enzyme-substrate complexes with less than \( n \) molecules of bound substrate may be neglected at any \([S]\) that is appreciable compared to \( K_s \). Under these conditions the rate equation will be given by:

\[
\frac{v}{V_{\text{max}}} = \frac{[S]^n}{K_{m, app} + [S]^n}
\]

\( (3.6) \)

where \( K_{m, app} = (a^n b^{n-2} c^{n-3} \ldots d^n) K_s^n = \sqrt{n[S]_{0.5}} \)

Equation-3.6 is known as the Hill equation. For a \( K \)-type homotropic allosteric enzyme a plot of \( v / V_{\text{max}} \) versus \([S]\) would be sigmoidal in nature with \( n \) being the value of \( v/V_{\text{max}} \) at the point of inflection. The value of \( n \) can also be obtained from the ratio of the substrate concentration at 90\% \( V_{\text{max}} \) to that at 10\% \( V_{\text{max}} \):

\[
\frac{[S]_{0.9}}{[S]_{0.1}} = \sqrt[9]{81}
\]

\( (3.7) \)

Equation 3.6 may be rearranged and written in the logarithmic form to give:

\[
\log \frac{v}{V_{\text{max}} - v} = n \log [S] - \log K_{m, app}
\]

\( (3.8) \)

A plot of \( \log v / (V_{\text{max}} - v) \) versus \( \log [S] \) will be a straight line with a slope of \( n \). This is known as the Hill plot.
Experimentally, the value of \( n \) is almost always less than the actual number of sites and the Hill plot deviates from linearity at low substrate concentrations. This is because when the cooperativity is not extremely high, at low substrate concentrations there are a significant number of enzyme molecules existing as enzyme-substrate complexes with less than \( n \) bound substrates. Under these conditions the rate equation does not reduce to the Hill equation. In such cases, the \( n \) in equations 3.6 to 3.8 should be designated \( n_H \) or \( n_{app} \). The value of \( n \) obtained experimentally from the rate curve or \([S]_0.9/[S]_0.1 \) ratio is essentially \( n_{app} \). On a Hill plot \( n_H \) (known as the Hill coefficient) is the slope at \( v = 0.5 \, V_{max} \). Thus, if the experimental data yields a \( n_{app} \) or \( n_H \) value of 1.8, it essentially means that the enzyme possesses at least 2 (the next highest integer above the \( n_{app} \) value represents the minimum number of actual sites) substrate binding sites with very strong cooperativity. However, it could also mean that there are four substrate binding sites with poor cooperativity. It is now recognized that \( n_H \) or \( n_{app} \) represents both the number of binding sites per enzyme and the degree of cooperativity between the sites. The closer the value of \( n_H \) or \( n_{app} \) to the actual number of binding sites, the greater the extent of interaction between sites.\(^{111}\) In literature the term “Hill-coefficient” is often used to refer to both \( n_H \) and \( n_{app} \).
Fig. 3-9 Scheme for a reaction catalyzed by a dimeric allosteric enzyme (V-system). E, S and P are the free enzyme, substrate and product, respectively. ES, SE and SES are the various enzyme-substrate complexes.

In a V-type homotropic allosteric enzyme, the substrate binding at one site increases the catalytic efficiency at the other site without affecting its intrinsic substrate dissociation constant. Such a system in a dimeric enzyme is shown in Fig. 3-9 (pg. 97). The SES complex is catalytically more efficient than the SE or ES complex by a factor $\beta$. In other words, each site in the dimeric SES complex is $\beta/2$ times more active than the same site in a monomeric ES complex. The velocity equation of such a system will be given by:

$$

\nu = \frac{2 [S] k_{cat} [E]_t}{K_S} + \frac{[S]^2}{K_S^2} \beta k_{cat} [E]_t 
\frac{1 + 2[S]}{K_S} + \frac{[S]^2}{K_S^2}

$$

or
where

\[ V_{\max} = \beta k_{\text{cat}} [E]_t \]  

(3.11)

\([E]_t = \text{total enzyme concentration}\]

At low substrate concentrations the observed velocity results mainly from the activities of the singly occupied SE and ES complexes. As the substrate concentration is increased, the more productive SES complex builds up. As a result, the rate curve will be sigmoidal. If there is no binding cooperativity between subunits, at any substrate concentration below saturation there will be a significant amount of enzyme existing as the ES or SE complex. Thus the kinetic equation for a purely \(V\)-type homotropic allosteric enzyme does not reduce to the Hill equation (eqn. 3.6). Hence, the experimentally measured value of \(n\) in such systems will always be the \(n_{\text{app}}\) discussed earlier.

Interactions that change both the intrinsic dissociation constant as well as catalytic efficiency are also possible. The rate equation for such a dimeric enzyme will be given by:

\[
\frac{v}{V_{\max}} = \frac{(2/\beta) [S]}{K_S} + \frac{[S]^2}{K_S^2} + \frac{[S]^2}{a K_S^2} 
\]

(3.12)
3.5 Previous Work Done

Initial work on UDPE was done by Kawamura et al. in late 1970s on the enzyme isolated from *E. coli* and *Bacillus cereus*. They showed that the optimal pH for the enzyme is between 7 and 9 and the purified enzyme is quite stable for several months. The enzyme activity is not affected by cofactors such as NAD$^+$, NADH, NADP$^+$ or NADPH up to 10 mM. Dithiothreitol (DTT) or 2-mercaptoethanol at 50 mM did not affect the activity of the enzyme indicating that there are no disulfide linkages within the enzyme that are required for its activity. However, unlike the UDPE isolated from *B. cereus*, the enzyme isolated from *E. coli* was strongly inactivated by *p*-chloromercuribenzoate, an organic mercurial that modifies the cysteine residues in the enzyme. The enzyme is almost 90% inactivated by 25 μM *p*-chloromercuribenzoate, but the activity is restored on treating the inactivated enzyme with DTT, indicating that there is probably an important cysteine residue which needs to be in the reduced form.

Kinetic studies by Kawamura et al. showed that the epimerase is allosterically regulated by its own substrate, UDP-GlcNAc. A plot of the rate of epimerization versus concentration of UDP-GlcNAc was sigmoidal with a Hill coefficient of 2.00 and an apparent $K_m$ value of 0.63 mM. At equilibrium, the reaction mixture contained UDP-GlcNAc and UDP-ManNAc in 10 : 1 ratio. For the reaction in the reverse direction, the epimerase showed an absolute requirement for UDP-GlcNAc. Using labeled UDP-[${}^{14}$C]ManNAc as substrate and unlabeled UDP-GlcNAc as the activator, it was shown that about 0.6 mM UDP-GlcNAc (approx. $K_m$ $app$ of UDP-GlcNAc) is required for half maximal activation of the enzyme. In the epimerase isolated from both *E. coli* and *B. aureus*, a plot of rate of epimerization versus concentration of UDP-[${}^{14}$C]ManNAc, in the presence of 0.5 mM UDP-GlcNAc, was a typical hyperbola. This
supports the fact that UDPE is allosterically regulated by UDP-GlcNAc but not by UDP-ManNAc. The value of $K_m$ for UDP-ManNAc in the epimerase isolated from *E. coli* was found to be 0.5 mM in the presence of 1 mM UDP-GlcNAc.

Other molecules like glucose, GlcNAc, GlcNAc 1-phosphate, GlcNAc 6-phosphate, UDP-GalNAc, UDP-glucose, ATP, UTP, UDP, UMP, NAD$^+$, and NADPH, all failed to activate the epimerase. However, UDP was found to be an inhibitor of the enzyme with a $K_t$ value of 1 mM. Interestingly, UDP did not change the values of $n_{app}$ or $K_{m,app}$ for UDP-GlcNAc suggesting a $V$-type or non-competitive inhibition (please refer to section 2.3, pg. 41 for a definition of non-competitive inhibition).

Later in 1993, Kiino et al. cloned the gene for UDPE (*nfrC* or *wecB* or *rffE*) from *E. coli* and inserted it into the vector, pET11a, to give the plasmid, pK86. The recombinant enzyme was isolated, purified and characterized by Morgan et al. It was found to be homodimeric with a subunit mass of 42,254 Da. Mechanistic studies on the enzyme showed that it catalyzes the epimerization reaction via anomeric C-O bond cleavage (Fig. 3-1, pg. 81). One line of evidence that supports the proposed mechanism is the release of intermediates by the epimerase upon extended incubation with its substrates. The intermediates, UDP and 2-acetamidoglucal, being thermodynamically much more stable than the substrates tend to accumulate in solution. However, since UDP is an inhibitor of the enzyme, as the intermediates accumulate the enzymatic activity falls off. The rate of intermediate release from an equilibrated mixture of substrates was measured by HPLC stopped assay, and it was found to be about 400 times slower than the rate of UDP-GlcNAc epimerization.
Fig. 3-10 Reaction catalyzed by UDPManNAc dehydrogenase

UDP-ManNAc dehydrogenase (UDPDH), which is used as a coupling enzyme to study the epimerase, catalyzes the two-fold oxidation of UDP-ManNAc to UDP-ManNAcUA (Fig. 3-10, pg. 101). In the process it reduces two molecules of NAD$^+$ to NADH. The enzyme is strictly specific for its substrates UDP-ManNAc and NAD$^+$. UDPDH requires a thiol-based reducing agent for its activity and shows maximum activation with 2 mM DTT. At an optimum pH of 10.0, it gives $K_m$ values of 0.38 mM and 0.21 mM for UDP-ManNAc and NAD$^+$, respectively. However, at pH 8.8, which is the pH for the coupled assay for the epimerase, UDPDH shows a value of 1.2 mM for the $K_m$ of UDP-ManNAc.$^{73}$

3.6 Experimental Procedures

3.6.1 Materials and General Procedures

(i) Materials

The bacterial strains JM109(DE3), MC4100 and XL1-blue supercompetent cells were obtained from Promega, New England Biolabs and Stratagene, respectively. The pKI86 plasmid that was used for the overexpression of wild-type UDPE and for all mutagenesis and cloning purposes was a gift from Drs. Lucia B. Rothman-Denes and Diane R. Kiino at the University of Chicago. The plasmid pUS01 used for the overexpression of the coupling enzyme UDP-
ManNAc dehydrogenase (UDPDH) was subcloned in our lab by Dr. Paul Morgan. The chemicals for preparing the LB media and the anion exchange resin DE52 were from Difco Laboratories. Protease inhibitors pepstatin and aprotinin were from Boehringer Mannheim. The dNTP mix and PWO polymerase used in all mutagenesis and subcloning experiments were from Gibco BRL and Roche Diagnostics, respectively. Antibiotics ampicillin and kanamycin were from Fisher Biotech and Calbiochem, respectively. All protein dialysis was done using Spectra/Por dialysis tubing (MWCO 12 – 14,000) from Spectrum Laboratories Inc. The disposable cuvettes used for kinetic assays were from Sarstedt.

The pET vectors used for subcloning of histidine-tagged wild-type and E117Q epimerase were from Novagen. The histidine-tagged proteins were purified on chelating Sepharose fast-flow resin from Pharmacia.

All PCR primers used for mutagenesis, cloning and sequencing were synthesized at the Nucleic Acid and Protein Services (NAPS) unit at University of British Columbia. The recombinant genes obtained from site-directed mutagenesis and sub-cloning were also sequenced at the NAPS unit.

All the chemicals used for the synthesis of UDP-ManNAc and buffers used for protein purification and storage were from Sigma-Aldrich.

(ii) General Procedures

Protein concentrations were determined by the method of Bradford. The Bradford reagent obtained from Bio-Rad was diluted five times to make a stock solution. Protein (5 µL) was added to the diluted Bradford reagent (995 µL). After mixing the solution by inverting the cuvette a couple of times, it was allowed to sit at room temperature for 5 min. The absorbance
of this solution at 595 nm was compared to a standard curve generated with solutions of BSA (bovine serum albumin from Bio-Rad) of known concentrations.

One unit of enzyme is defined as the amount of enzyme required to convert 1 μmole of substrate into product in 1 min.

Plasmids were purified using the Wizard™ Minipreps Plasmid DNA Purification system from Promega. PCR products were purified using the SpinPrep™ PCR clean-up kit from Novagen. The concentrations of double stranded plasmids were determined from their absorbance at 260 nm using the following formula:

\[ \chi (\mu g/mL) = A_{260} \times 50 \]

The concentrations of primers were also determined from their absorbance at 260 nm using the following formula:

\[ \chi (\text{picomole/μl}) = A_{260} \times \frac{100}{1.5N_A + 0.7N_C + 1.2N_G + 0.84N_T} \]

where \( N_A, N_C, N_G, \) and \( N_T \) are the number of adenosine, cytidine, guanosine and thymidine bases in the primer.

Unless otherwise stated, proteins were always handled at 4 °C and stored at -80 °C in buffers containing 10% glycerol. The general techniques in molecular biology used were those described in “Short Protocols in Molecular Biology”.95
3.6.2 Site-Directed Mutagenesis

The plasmids for mutants of UDPE were constructed using the QuickChange™ Site-directed mutagenesis kit (QSDM) from Stratagene. The pKI86 plasmid (6.8 kb, pET11a vector containing the nfrC gene) was amplified using a pair of complementary primers containing the desired mutation(s) (Table 3-1, pg. 105). Each primer (28 to 33 base pairs long) was designed such that its melting temperature ($T_m$) was $> 68 \, ^\circ C$ as calculated using the formula:

$$T_m = 81.5 + 0.41 \times (%GC) - 675/N - \% \text{mismatch} \quad \{N = \text{primer length}\}$$

where "N" is the primer length and "%GC" is the percent content of guanosine and cytosine bases.

The primers were further checked for formation of hairpin loop using the program OLIGO before submitting the primer sequence for synthesis at NAPS unit at the University of British Columbia.

The PCR (Polymerase Chain Reaction) reaction mixture for amplification of the plasmid contained 5 μL of 10 x polymerase buffer, 5 μL of 2.5 mM dNTP mix, 50 picomole of each primer, 20 ng of pKI86 template plasmid, and 2.5 units of PWO polymerase in a total volume of 50 μL. The polymerase was added last to prevent its endonuclease activity from cleaving the template DNA. The reaction mixture was topped with 30 μL of mineral oil to prevent evaporation and passed through the following cycles in an M J Research minicycler: one cycle of 1 min at 94 °C, 20 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 5 min at 72 °C, followed by one cycle of 7 min at 72 °C. After amplification, the entire PCR reaction mixture was incubated at 37 °C for 1 hr with 10 units of DpnI restriction enzyme to digest the template DNA. The PCR
product (4 μL) was then transformed into 100 μL MC4100 or XL1-Blue supercompetent cells and plated on an LB agar plate containing 50 μg/mL ampicillin.

Table 3-1 Primers used for site-directed mutagenesis and sequencing. The bases altered for introducing the mutation are underlined.

<table>
<thead>
<tr>
<th>Primers for D95N mutation</th>
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<tbody>
<tr>
<td>JS23: 5'- CGT GTT CAC GGC AAT ACG ACG ACG ACG -3'</td>
</tr>
<tr>
<td>JS24: 5'- CGT CGT CGT CGT ATT GCC GTG AAC CAG C -3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primers for E117Q mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>JS25: 5'- GGT CAC GTT CAG GCT GGT CTG CGC ACG -3'</td>
</tr>
<tr>
<td>JS26: 5'- GCC CGT GCG CAG ACC AGC CTG AAC GTG -3'</td>
</tr>
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</table>

<table>
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<tr>
<th>Primers for E131Q mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>JS27: 5'- CGC CGT GGC CGC AAG AGG CTA ACC G -3'</td>
</tr>
<tr>
<td>JS28: 5'- GTA CGG TTA GCC TCT TGC GGC CAC GG -3'</td>
</tr>
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</table>

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<tr>
<th>Primers for H213N mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>JS29: 5'- CTG GTG ACC GGT AAT ACG ACG GAG AG -3'</td>
</tr>
<tr>
<td>JS30: 5'- CCG AAA CTC TCA CGC CTG TTA CCG GTC -3'</td>
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<tr>
<th>Primers for K15A mutation</th>
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</thead>
<tbody>
<tr>
<td>JS33: 5'- CGC CCG GAA GCC ATC GCG ATG G -3'</td>
</tr>
<tr>
<td>JS34: 5'- CCA TCG CGA TGG CTT CCG GGC G -3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primers for sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>JS31: 5'- CCG GCCATC TGGCGA TGT ATC ACT TCT C -3'</td>
</tr>
<tr>
<td>JS35: 5'- CAG CGC GTG GCA GAT TTC TTC -3'</td>
</tr>
</tbody>
</table>
Single colonies from the plate were used to induce 10 mL LB media containing 50 μg/mL ampicillin. The cells were allowed to grow overnight at 37 °C to an optical density (OD₆₀₀) of 1.8 and harvested by centrifugation at 5000 rpm for 5 min. The plasmids were isolated and purified using the Wizard™ Minipreps Plasmid DNA Purification system and submitted to the NAPS unit for sequencing. The sequences were manually checked for the introduction of the correct mutation and presence of any undesired mutation.

3.6.3 Overexpression and Purification of WT and Mutant UDPE

Wild-type (WT) and mutant UDPE were overexpressed in E. coli JM109 (DE3) cells bearing their respective plasmids. The cells containing the plasmid of interest were first grown at 37 °C on an LB agar plate containing 50 μg/mL ampicillin. Single colonies from the plate were used to inoculate 4 x 500 mL LB media containing 50 μg/mL ampicillin. The cells were allowed to grow to an OD₆₀₀ (optical density at 600 nm) of 0.8 at which time they were induced for overexpression with 100 μg/mL isopropylthio-β-galactoside (IPTG) and allowed to continue growth to an OD₆₀₀ of 1.6 - 1.8. The cells were then harvested by centrifugation at 5000 rpm in a GSA rotor (Sorval), resuspended in lysis buffer (50 mM TEA-HCl, pH 7.0, containing 10% glycerol, 2 mM DTT, 1μg/mL pepstatin and 1 μg/mL aprotinin) and lysed by passage through an ice-cooled French press at 20,000 psi. Cell debris was removed by centrifugation at 6000 rpm for 20 min and the supernatant was brought to 40% ammonium sulfate saturation. The precipitate was removed by centrifugation at 5000 rpm for 30 min and the supernatant was brought from 40% to 60% ammonium sulfate saturation. The precipitate obtained between 40% - 60% ammonium sulfate saturation contained the epimerase. It was separated by centrifugation (5000 rpm for 30 min) and re-dissolved in about 10 mL buffer-A (50 mM TEA-HCl, pH 7.7,
containing 10% glycerol and 4 mM DTT). The protein was dialyzed overnight against 1 liter buffer-A to remove ammonium sulfate.

The protein from overnight dialysis was loaded on a 50 mL DE-52 column equilibrated with buffer-A. After washing the column with 4 CV (column volumes) of buffer-A, and 2 CV of 0.06 M NaCl in buffer-A, the protein was eluted with 2 CV of 0.16 M NaCl in buffer-A. The fractions containing the enzyme were concentrated (if required) using another 5 mL DE-52 column, equilibrated with buffer-A. The fractions pooled from the first column were diluted 5 times with fresh buffer-A to reduce the NaCl concentration. This solution was loaded on the 5 mL column and eluted with 0.3 M NaCl in buffer-A. This method was much faster than using centrifugal filter devices and at the same time loss of protein during concentration was also minimized. The concentrated protein was dialyzed against 2 x 1 liter fresh buffer-A and stored at -80 °C.

Immediately prior to the next step, the protein solution was thawed and filtered through a 0.2 μm syringe filter. About 40 mg of the protein was injected into a 5 mL Hi-Trap Q-Sepharose High Performance anion exchange column (Pharmacia) pre-equilibrated with buffer-A. After washing the column with the same buffer, the epimerase was eluted with a linear gradient of 0.08 – 0.15 M NaCl in buffer-A. Fractions containing the pure epimerase (indicated by a single band on 12% SDS-PAGE) were pooled and stored at -80 °C.

3.6.4 Overexpression and Purification of UDP-ManNAc Dehydrogenase (UDPDH)

UDPDH was overexpressed using pUS01 plasmid in JM109(DE3) cells. The purification procedure for UDPDH was the same as that used for UDPE. However, after the final
purification step, UDPDH was dialyzed against 50 mM TEA-HCl buffer (pH 8.8, containing 10% glycerol and 4 mM DTT) and stored at -80 °C.

3.6.5 Epimerase Assay 1 (Coupled Assay)

The ability of the WT and mutant enzymes to catalyze the epimerization of UDP-GlcNAc to UDP-ManNAc (forward reaction) was studied by a continuous assay using UDPDH as the coupling enzyme. Each assay mixture contained (total volume 1 mL) 50 mM Tris-HCl (pH 8.8), 2 mM DTT, 4.5 mM NAD$^+$, 5 mg UDPDH and variable concentrations of UDP-GlcNAc. The reaction mixture was monitored for 5 min to ensure that the background rate was negligible and then the epimerization reaction was initiated by adding 5.0 x 10$^{-3}$ units of the epimerase. The rate data obtained from these runs were directly fit into the Hill equation using the computer program Grafit.

3.6.6 Epimerase Assay 2 (Stopped Assay)

The epimerization reactions in both directions were monitored by stopped assay using HPLC. The substrate and the epimerase were mixed in 50 mM Tris-HCl buffer (pH 8.8, containing 2 mM DTT) and incubated in a water bath at 37 °C. Aliquots were taken at different time points, diluted to give a final UDP-sugar concentration of 0.1 mM and frozen in liquid nitrogen. Just prior to injection, the aliquots were thawed and 40 µL was applied to an ion-paired reverse phase HPLC column (Waters Radial-Pak 8NVC18) pre-equilibrated with phosphate buffer (50 mM, pH 7.0, containing 2.5 M TBAHS). The components were separated with the same buffer-A and monitored at 262 nm. The % conversion was determined from the relative peak area.
3.6.7 UDP Release Assay

The rate of UDP release was determined by both stopped assay and coupled assay. The stopped assay for UDP release was the same as the epimerization assay 2 mentioned in section 3.6.6. Control samples containing heat denatured epimerase were run to ensure that the sugar nucleotides did not non-enzymatically hydrolyze to give UDP.

The rate of UDP release by the epimerase was also determined by a coupled assay modified from that given in literature (Fig. 3-22, pg. 139). Each assay mixture contained 20 mM HEPES (pH 7.8), 50 mM KCl, 5 mM MgCl₂, 5 mM DTT, 2 mM PEP, 0.15 mM NADH, 200 units of pyruvate kinase, 377 units of lactate dehydrogenase and 8 mM UDP-GlcNAc in a total volume of 1 mL. After checking for any background rate for 5 min, the reaction was initiated by adding 100 μg/mL epimerase in the case of the wild-type enzyme and 1 mg/mL of the mutants. The rate of UDP release was measured by following the decrease in absorbance at 340 nm (consumption of NADH) at 37 °C.

3.6.8 Subcloning of Histidine-Tagged Wild-Type and E117Q Epimerase

The genes for WT and E117Q epimerase were subcloned from plasmids pKI86 and pJT03, respectively, into the pET-30 Xa/LIC vector using the ligation-independent cloning technique. The steps involved were as follows:
(i)  **Amplification of nfrC gene.**

The genes were amplified using specially designed primers which would create the overhangs required for subcloning. The sequences of the forward and reverse primers were as follows:

<table>
<thead>
<tr>
<th>Primer (forward):</th>
<th>5' - GGT ATT GAG GGT CGC ATG AAA GTA CTG ACT GTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overhang</td>
<td>nfrC gene sequence</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer (reverse):</th>
<th>5' - AGA GGA GAG TTA GAG CCT TAT AGT GAT ATC C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overhang</td>
<td>nfrC gene sequence</td>
</tr>
</tbody>
</table>

The PCR reaction mixture (total volume 50 μL) contained 5 μL of 10 x polymerase buffer, 5 μL of 2.5 mM dNTP mix, 20 ng template plasmid, 50 picomole each of the forward and reverse primers and 0.5 μL PWO polymerase. The gene was amplified using the following cycles: one cycle of 1 min at 94 °C, 20 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 50 sec at 72 °C, followed by one cycle of 7 min at 72 °C. The PCR product was purified using SpinPrep™ PCR clean-up kit to remove any dNTPs left over from the PCR reaction. The removal of all dNTPs is important since they could interfere with the T4 DNA polymerase treatment in the next step.

(ii)  **Preparation of insert**

The purified PCR product was treated with T4 DNA polymerase to create overhangs required for annealing it to the vector. The reaction mixture containing 0.2 picomole PCR product, 1 unit T4 DNA polymerase, 2.5 mM dGTP and 5 mM DTT in T4 DNA polymerase
buffer (20 μL total volume) was incubated at 22 °C for 30 min followed by 75 °C for 20 min. In the presence of dGTP only, the 3’ to 5’ exonuclease activity of the polymerase removes the nucleotides of the PCR product until it encounters a guanidine residue in the chain. By virtue of the sequence of the primers which were used for amplification, the polymerase treatment creates overhangs in the insert that complements the overhangs present on the LIC vector.

(iii) **Annealing and transformation**

The pET-30 Xa/LIC vector (Novagen) was supplied pre-treated with T4 DNA polymerase. For the annealing step 0.01 picomole of the vector and 0.02 picomole of polymerase treated insert were incubated at 22 °C for 5 min. At this point, 6.25 mM EDTA was added to the mixture and incubated at 22 °C for another 5 min. The complete mixture of the insert annealed to the vector (total volume 4 μL) was immediately transformed into *E. coli* MC4100 and plated on an LB-agar plate containing 100 μg/mL kanamycin. The efficiency of transformation was very low and only one or two colonies were obtained.

(iv) **Isolation of plasmid and sequencing**

Single colonies from the plate were used to inoculate 10 mL LB media containing 100 μg/mL kanamycin. The cells were allowed to grow to an OD₆₀₀ of about 1.8 and the plasmids from the cells were isolated and purified using Wizard™ Minipreps Plasmid DNA purification kit. The purified plasmids were submitted for sequencing using primers JS31 and JS35 (Table 3-1, pg. 105). The sequences were manually checked for presence of the tags and any unwanted mutations.
3.6.9 Overexpression and Purification of Histidine-Tagged Wild-Type and E117Q Mutant UDPE

The histidine-tagged wild-type (His-WT) and E117Q (His-E117Q) UDPE were overexpressed in *E. coli* JM109 (DE3) using the plasmids pJS22 and pTB11, respectively. After transformation, the cells were allowed to grow overnight at 37 °C on an LB-agar plate containing 100 µg/mL kanamycin. Single colonies from the plate were used to inoculate 4 x 500 mL LB media containing 100 µg/mL kanamycin. The cell culture was allowed to grow at 37 °C to an OD$_{600}$ of 0.8 at which point overexpression of the protein was induced with 70 µg/mL IPTG. The cells were allowed to continue their growth up to an OD$_{600}$ of 1.8 and then harvested by centrifugation at 5000 rpm in a GSA rotor (Sorval). The cell pellet was resuspended in TEA-HCl buffer (50 mM, pH 7.7 containing 10% glycerol, 2 mM DTT, 1µg/mL aprotinin and 1 µg/mL pepstatin) and the cells were lysed by passage through an ice-cooled French press at 20,000 psi. The cell debris was removed by centrifugation at 5000 rpm for 20 min and the supernatant was directly loaded on to a DE52 column pre-equilibrated with TEA-HCl buffer (50 mM, pH 7.7 containing 10% glycerol and 2 mM DTT). After washing the column with fresh buffer, the protein was eluted with 0.16 M NaCl in the same buffer. Fractions containing the epimerase were pooled and dialyzed overnight against 1 liter binding buffer (20 mM Tris-HCl, pH 7.9, containing 5 mM imidazole and 0.5 M NaCl). (Note: if the enzyme was to be stored at -80 °C at this stage, 10% glycerol was added to the buffer).

A 50 mL column of chelating Sepharose fast-flow resin (Pharmacia) was first washed with 4 CV (column volumes) of distilled water and then charged with 4 CV 50 mM NiSO$_4$ solution. The column was then equilibrated with 5 CV binding buffer (20 mM Tris-HCl, pH 7.9,
containing 5 mM imidazole and 0.5 M NaCl) and the protein from the DE52 column (dialyzed against binding buffer) was loaded on to the column. After washing the column with 1 CV binding buffer and 3 CV wash buffer (20 mM Tris-HCl, pH 7.9, containing 100 mM imidazole and 0.5 M NaCl), the protein was eluted with 2 CV of the elution buffer (20 mM Tris-HCl, pH 7.9, containing 1 M imidazole and 0.5 M NaCl). Fractions containing the epimerase were pooled, dialyzed against 2 x 1 liter TEA-HCl buffer (50 mM, pH 7.7 containing 10% glycerol and 4 mM DTT) and stored at -80 °C.

3.6.10 Synthesis of UDP-N-Acetylmannosamine

(i) 1-Bromo-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-mannopyranose (1)

ManNAc (1.0 g, 4.6 mmoles) was protected by peracetylation using a 1:2 mixture of acetic anhydride (20 mL) and pyridine (40 mL). The reaction mixture was allowed to stir at room temperature for 1 hr. At the end of the reaction (as indicated by TLC (solvent: ethyl acetate)) the solvents were evaporated under vacuum. The product was dissolved in CH$_2$Cl$_2$, extracted twice with saturated NaHCO$_3$ to remove any traces of acetic acid and then washed with water. The organic layer containing the product was dried over anhydrous MgSO$_4$ and rotary evaporated to dryness to give the crude 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-D-mannopyranose (or peracetylated ManNAc) as a white powder.

The crude peracetylated ManNAc (1.4 g, 3.5 mmoles) was dissolved in CH$_2$Cl$_2$ (25 mL) and HBr (in 35% acetic acid) (5 mL). The reaction mixture was stirred for 3 hrs at room temperature and the solvent was rotary evaporated to concentrate it to about 3 - 5 mL. This concentrated reaction mixture was loaded onto an alumina (neutral) column equilibrated with 99:1 CHCl$_3$/Py. The product was eluted with the same solvent and the fractions containing pure
1-bromo-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-mannopyranose (1) (as indicated by TLC (solvent: ethyl acetate)) were pooled and rotary evaporated to dryness to obtain a white solid in 40% yield. $^1$H NMR (CDCl$_3$) $\delta$ 6.0 (s, 1H), 5.9 (d, 1H), 5.1 (dd, 1H), 4.6 (m, 1H), 4.2 (dd, 1H), 4.0 (m, 2H), 2.0 – 2.2 (m, 12); DCI-MS 409 (M + H$^+$).

(ii) 4,5-(3,4,6-Tri-O-acetyl-2-deoxy-D-mannopyranosyl)-2-methyl-$\Delta^2$-oxazoline (2)

1-Bromo-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-mannopyranose (1) (400 mg, 0.96 mmoles) was dissolved in 10 mL acetonitrile. K$_2$CO$_3$ (276.4 mg, 2 mmoles) was added to the solution and the reaction mixture was allowed to stir at room temperature for two hours. The K$_2$CO$_3$ was removed by filtration and the solvent was removed by evaporation. The pure 4,5-(3,4,6-tri-O-acetyl-2-deoxy-D-mannopyranosyl)-2-methyl-$\Delta^2$-oxazoline (2) was crystallized from ethyl acetate/hexane (90% yield). $^1$H NMR (CDCl$_3$) $\delta$ 5.8 (d, 1H), 5.3 (dd, 1H), 5.0 (dd, 1H), 4.4 (m, 1H), 4.1 (m, 2H), 3.8 (m, 1H), 2.0 – 2.2 (m, 12); DCI-MS 330 (M + H$^+$).

(iii) ManNAc $\alpha$-1-phosphate (3)

The ManNAc 1-phosphate (3) was prepared form the oxazoline derivative (2) by a procedure slightly modified from that given in the literature.$^{114}$ The oxazoline derivative (2) (92 mg, 0.28 mmoles) and 1.1 equivalents of dibenzylphosphate (DBP) were dissolved in dichloroethane (DCE) (10 mL). The reaction mixture was stirred at room temperature in the presence of 4 Å molecular sieves and under argon atmosphere for 1 hr. After removing the molecular sieves by filtration the reaction mixture was diluted with methanol (5ml) and immediately hydrogenated under 1 atm of H$_2$ with 10% Pd/C (100 mg). Debenzylation was followed by TLC (CHCl$_3$ : MeOH : H$_2$O : 5 : 5 : 1). The peracetylated ManNAc $\alpha$-1-phosphate was purified by preparative TLC using CHCl$_3$ : MeOH : H$_2$O / 5 : 5 : 1 as the solvent system. The desired band from the preparative TLC plate was removed, dried and the product extracted
into methanol (15 mL). The peracetylated ManNAc α-1-phosphate was immediately deacetylated by adding freshly prepared 1 M sodium methoxide in methanol until the resulting solution was basic by pH paper. The solution was allowed to sit at room temperature until the reaction was complete as judged by TLC (solvent- CHCl₃ : MeOH : H₂O / 5 : 5 : 1). The product (3) was neutralized by passing it through cation exchange resin (IR 120 plus, Aldrich) (10 mL) in the pyridinium form. The ¹H-NMR spectrum of (3) was in agreement with that reported in literature.¹²³

(iv) UDP-N-acetylmannosamine

ManNAc α-1-phosphate was coupled to UMP-morpholidate using the modified Khorana-Moffat procedure mentioned in literature.¹²² ¹H-NMR spectrum of the final product was in agreement with that reported in literature.

3.6.11 Circular Dichroism of Wild-Type and Mutant Enzymes

CD spectra were acquired in the far-UV region (190-240 nm) at 25 °C on a Jasco J-810 spectropolarimeter. Each sample contained 0.25 mg/mL of the enzyme in phosphate buffer (50 mM, pH 7.0). The spectra were converted to molar elipticity using the program Spectra Manager version 1.5.2.01.

3.6.12 Fluorescence Studies

All fluorescence spectra were taken on a Cary Eclipse fluorescence spectrophotometer controlled by Cary 1.0 software. The measurements were taken at room temperature in 50 mM Tris-HCl buffer (pH 8.8) containing 2 mM DTT. For characterization of the wild-type and mutant epimerases, solutions containing 0.5 mg/mL of the enzyme were excited with 295 nm wavelength light and fluorescence was recorded between 310 and 420 nm.
For binding studies with the wild-type epimerase the tryptophan residues were excited at 295 nm and the emission was recorded at 335 nm. The enzyme concentration was 0.5 mg/mL (5.75 μM dimer concentration) and the UDP-GlcNAc concentration was varied from 0.01 mM to 2.0 mM. Corrections were made in the calculation of the substrate concentration to account for the fact that the actual concentration of UDP-GlcNAc in the cuvette containing the wild-type epimerase is only 91% of the added amount due to epimerization. The fluorescence of the enzyme in the absence of substrates (F₀) was subtracted from the fluorescence of the enzyme in the presence of substrates (F) to give the net increase in fluorescence (δF) on binding of the ligand. The number of binding sites per dimer and the dissociation constant were determined using a Scatchard plot. The equation for the Scatchard plot is given by:

\[
\frac{B}{L} = nk - kB
\]  \hspace{1cm} (3.13)

where,

\[
B = \frac{\text{moles of bound ligand}}{\text{total moles of receptor}} = \frac{\delta F}{F_0}
\]

\[
n = \text{number of binding sites on the receptor}
\]

\[
k = \text{binding affinity} = 1/K_d
\]

\[
L = \text{concentration of free UDP-GlcNAc}
\]
3.7 Results & Discussion

3.7.1 Site-directed Mutagenesis of Active-Site Residues

The crystal structure of UDPE (2.5 Å) complexed with its substrate is known. In the final crystal structure only the UDP portion of the substrate is visible. An interesting feature of the structure of UDPE is that the two subunits of the homodimeric enzyme show two different conformations. One subunit shows a 10° interdomain rotation as compared to the other subunit (Fig. 3-11A, pg. 118). The conformational difference between the two subunits has been implicated in the allosteric regulation of the enzyme. Another interesting feature is that UDPE is structurally homologous to members of a superfamily of glycosyltransferases that includes β-glucosyltransferase, glycogen phosphorylase, etc. (Please refer to section 1.3.2.2b, pg. 18 for more details).

The active site of the epimerase, as indicated by this bound UDP, lies in the interdomain cavity. Quite a few residues in the active site are conserved among UDPE isolated from different sources. On the basis of the crystal structure and sequence homology, five active site residues were targeted for mutagenesis studies: Lys15, Asp95, Glu117, Glu131 and His213 (Fig. 3-11B, pg. 118). In a structure guided sequence alignment of UDPE from different sources, all these residues have been found to be completely or partially conserved. Thus while Asp95 and His213 have been strictly conserved in all 29 sequences, Glu117 and Glu131 have been conserved as an Asp or Glu, and Lys15 has been conserved as a Lys or Arg in at least 27 of the 29 sequences. All these residues are found in the region close to where the GlcNAc portion of UDP-GlcNAc is expected to bind. The position of these five residues in the active site (indicated by UDP) of the open and closed subunits is shown in Fig. 3-11B (pg. 118).
distance between the positions of the five residues in the two subunits is given in Table 3-2 (pg. 119). We see that there is considerable movement in the positions of His213 and Glu131. No significant movement has been observed in the positions of other residues. These relative positions might play an important role in cooperativity.

Fig. 3-11 Crystal structure of UDPE. (A). The two subunits are overlapped to show the interdomain rotation. The subunit in green is in the open conformation and the subunit in red is in the closed conformation. (B). Active site residues selected for site-directed mutagenesis and the bound UDP. Again the positions in the open (green) and closed (red) conformations are shown. H-bonds are shown by dotted lines.
Table 3-2 Distance between position of residues in the closed and open subunits

<table>
<thead>
<tr>
<th>Residues</th>
<th>Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys15 NZ</td>
<td>2.5</td>
</tr>
<tr>
<td>Asp95 OD1</td>
<td>2.4</td>
</tr>
<tr>
<td>Glu117 OE2</td>
<td>1.3</td>
</tr>
<tr>
<td>Glu131 OE2</td>
<td>3.4</td>
</tr>
<tr>
<td>His213 NE2</td>
<td>4.9</td>
</tr>
<tr>
<td>UDP O3B</td>
<td>1.7</td>
</tr>
</tbody>
</table>

It should be noted that His 213 is found to be H-bonded to the oxygen of the β-phosphorus in the closed subunit, while in the open subunit it is about 7Å away (Fig. 3-11B, pg. 118). It has been proposed that His213 could act as a general acid catalyst to activate the UDP of the substrate for elimination. The structural homolog of Lys15 in UDPE is Glu22 in β-glucosyltransferase (BGT). This residue has been proposed to be the catalytic base in BGT. In UDPE, Lys15 forms a salt bridge with Glu117 in the closed subunit. This could be significant in maintaining the charge state of both Lys15 and Glu117. Lys15 lies close to the β-phosphate of UDPE and could play an important role in binding the negative charge of the phosphate group of the substrate. Glu117 which likely exists in the deprotonated form (due to a H-bond with Lys15) could play an important role in deprotonating/reprotonating the C-2 position of one of the substrates. To study the role of these residues in catalysis, allostery and/or substrate binding, the glutamates were conservatively mutated to glutamines, aspartate and histidine were changed into asparagines, and lysine was changed into alanine.

The mutations were introduced into the nfrC gene (Appendix-3) contained in the plasmid pKI86 using Stratagene’s QuickChange Site-directed Mutagenesis (QSDM) kit. The
protocol for QSDM is based on the fact that DNA isolated from most *E. coli* strains is dam-methylated (*N*-position of doxy-adenosine in the sequence GATC is methylated) while the PCR amplified DNA is not methylated. Hence, the template DNA used for amplification can be selectively digested by the restriction enzyme DpnI. This eliminates the need for linearization of DNA and the recombination steps required in the more conventional mutagenesis techniques. The QSDM method is also more reliable since the chances of contamination of the mutant plasmid with the wild-type template plasmid are greatly reduced with the DpnI digestion step. Furthermore, this method also gives high efficiency in transformation. The mutant genes were fully sequenced to check for the introduction of the desired mutation and to eliminate the possibility of any unwanted mutation. The plasmids containing the mutations K15A, D95N, E117Q, E131Q and H213N were named pJT01, pJT02, pJT03, pJT04 and pJT05, respectively.

3.7.2 Isolation and Purification of UDP-GlcNAc 2-Epimerase and UDP-ManNAc Dehydrogenase

UDPDH (UDP-ManNAc dehydrogenase) and wild-type and mutant UDPE (UDP-GlcNAc 2-epimerase) were overexpressed in *E. coli* by procedures mentioned in the experimental section. The plasmid pKI86 used for overexpression of UDPE was obtained from Drs. L. B. Rothman-Denes and D. R. Kiino at the University of Chicago. They had cloned the *nfrC* gene (gene that codes for UDP-GlcNAc 2-epimerase) from *E. coli* into the pET11a vector. The plasmid pUS01 used for the overexpression of UDPDH was previously constructed in our lab by Dr. Paul Morgan. The *rfdD* gene that encodes UDPDH was subcloned from the plasmid pCA62 (obtained from Dr. P. D. Rick) into the pET11a vector. (Please refer to Appendix-2 for a description of the over-expression of proteins using pET vectors). Plasmids used for the overexpression of mutant epimerases were constructed from pKI86 by site-directed
mutagenesis. The overexpression of all proteins was achieved by transforming the respective plasmid into *E. coli* JM109 (DE3).

<table>
<thead>
<tr>
<th>Mol. Wt std</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>66 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 3-12 SDS-PAGE showing the purified proteins.** Lane 1: UDPDH; lane 2: WT UDPE; lane 3: K15A; lane 4 D95N; lane 5: E117Q; lane 6: E131Q; lane 7: H213N

The purification procedures for UDPDH, UDPE and its mutants were modified from that mentioned in the literature. UDPDH purified by the procedure mentioned in literature gave a background rate of 15 – 20% of the epimerase *V*<sub>max</sub> in kinetic assays. This could have been due to some impurities in the UDPDH enzyme preparation. To overcome this problem, a new step of ammonium sulfate precipitation (40 – 60% ammonium sulfate saturation) was introduced in the purification procedures for both the epimerase and the dehydrogenase. The pH of the buffer used for purification by anion exchange columns was also changed. These changes helped in achieving greater than 95% purity of the enzymes (as indicated by SDS-PAGE) (Fig. 3-12, pg. 121) and the UDPDH thus prepared did not give any background rate. The mutant epimerases behaved similarly to the wild-type epimerase during all purification procedures.
While the purified UDPE stored at -80 °C was stable and did not show any significant loss of activity over several months, UDPDH tended to lose its activity over a period of a couple of months.

3.7.3 Characterization of Purified Proteins

All the purified proteins were characterized by ESI-MS (electrospray ionization mass spectrometry). The purified UDPDH showed the expected mass of 45,718 ± 7 and the wild-type epimerase showed a mass of 42,254 ± 7. All mutants behaved similar to the wild-type during the purification procedures and showed the expected mass (Table 3-3, pg. 122).

Table 3-3 Calculated and observed mass of WT and mutant UDPE

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Calculated mass (Da)</th>
<th>Observed mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>42,254</td>
<td>42,259 ± 7</td>
</tr>
<tr>
<td>D95N</td>
<td>42,253</td>
<td>42,260 ± 7</td>
</tr>
<tr>
<td>E117Q</td>
<td>42,253</td>
<td>42,260 ± 7</td>
</tr>
<tr>
<td>E131Q</td>
<td>42,253</td>
<td>42,260 ± 7</td>
</tr>
<tr>
<td>K15A</td>
<td>42,196</td>
<td>42,199 ± 7</td>
</tr>
<tr>
<td>H213N</td>
<td>42,233</td>
<td>42,234 ± 7</td>
</tr>
</tbody>
</table>

An interesting observation was that both WT and mutant proteins are prone to dimerization through disulfide linkages with time. This is not surprising as in the crystal structure, the thiols of Cys72 of adjacent subunits lie within 4Å of each other. But since the crystallization was done under reducing conditions, no intersubunit disulfide linkage was found between the two Cys72 residues. A mass spectrum of the E117Q mutant showing a mixture of
monomeric and dimeric protein is shown in Fig. 3-13 (pg. 125). As compared to the WT epimerase, the mutant enzymes were more prone to dimerization. A check of the activity of the enzymes showed that this dimerization did not affect their catalytic ability to any significant extent and could be easily reversed by adding extra DTT to the solution containing the dimerized proteins. For this reason, the amount of DTT in all enzyme storage buffer solution was increased from 2mM (as reported in literature) to 4mM.

The wild-type and mutant epimerases were further characterized by circular dichroism (CD) and fluorescence spectroscopy. The CD of the enzymes between 190 to 240 nm showed that all mutants were properly folded (Fig. 3-14, pg. 125). However, the E117Q mutant did show a slight decrease in molar elipticity as compared to the WT epimerase. The fact that this decrease in molar elipticity was not a result of an error in determining the enzyme concentration by Bradford assay was confirmed by checking the absorbance of the enzyme solution at 280 nm. The fluorescence spectrum between 310 to 420 nm shows changes in the conformation of the wild-type and mutant epimerases (Fig. 3-15, pg. 126). There are four tryptophan residues in UDPE that could fluoresce at this wavelength. One of them (Trp129) lies at the dimer interface and another (Trp179) in the interdomain region. Any change in the conformation of the dimeric enzyme could thus result in a change in its native fluorescence. The different tendencies of the epimerases to dimerize through disulfide linkages and the slight variations in the fluorescence of the enzymes indicate some variation in the conformation of the mutants at the dimer interface.
Fig. 3-13 Mass spectra of E117Q epimerase showing (A) mixture of monomeric and dimeric enzyme (B) mass of monomeric enzyme (C) mass of dimeric enzyme.

Fig. 3-14 CD spectra of WT and mutant UDPE
Fig. 3-15 Fluorescence spectra of the wild-type and mutant epimerases.

3.7.4 Kinetics of the Forward Reaction in the Wild-Type and Mutant Epimerases

The epimerization reaction catalyzed by UDPE was assayed in the UDP-GlcNAc to UDP-ManNAc direction using UDP-ManNAc dehydrogenase (UDPDH) as the coupling enzyme (Fig. 3-16, pg. 126). The dehydrogenase oxidizes the UDP-ManNAc produced by UDPE to UDP-\(N\)-acetylmannosaminuronic acid (UDP-ManNAcUA) with the concurrent reduction of two molecules of NAD\(^+\). The rate of epimerization was determined by following the increase in absorbance at 340 nm (NADH produced) at 37 °C and dividing it by a factor of two to account for the stoichiometry of the reaction.

Fig. 3-16 Coupled assay for determining the rate of epimerization of UDP-GlcNAc by UDPE
Table 3-4 Kinetics of WT and mutant UDPE in the epimerization of UDP-GlcNAc

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat, app}}$ (sec$^{-1}$)</th>
<th>$K_m, \text{ app}$ (mM)</th>
<th>$n_{\text{app}}$</th>
<th>$k_{\text{cat}}/K_{m, \text{ app}}$ (sec$^{-1}$mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.1 $\pm$ 0.3</td>
<td>0.6 $\pm$ 0.06</td>
<td>1.8 $\pm$ 0.1</td>
<td>14.2</td>
</tr>
<tr>
<td>H213N</td>
<td>0.15 $\pm$ 0.03</td>
<td>17.2 $\pm$ 0.7</td>
<td>1.3 $\pm$ 0.02</td>
<td>$8.7 \times 10^{-3}$</td>
</tr>
<tr>
<td>K15A</td>
<td>-</td>
<td>$&gt;&gt;100$</td>
<td>-</td>
<td>$1.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>E117Q</td>
<td>$\sim 4 \times 10^{-4}$</td>
<td>$&lt;3.0$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E131Q</td>
<td>$\sim 4 \times 10^{-4}$</td>
<td>$&lt;3.0$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D95N</td>
<td>$\sim 3 \times 10^{-4}$</td>
<td>$&lt;3.0$</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The errors reported here are the standard deviations of the points that make the rate versus [substrate] curve. Each experiment was repeated three times and the results were found to be reproducible within a 10% error margin.

In the case of the WT epimerase, a plot of rate versus concentration of UDP-GlcNAc gave a sigmoidal curve with a $n_{\text{app}}$ value of 1.8 $\pm$ 0.1 (Table 3-4, pg. 127, Fig. 3-17, pg. 128). The sigmoidal nature of the rate dependence on substrate concentration indicates that UDP-GlcNAc allosterically regulates the activity of the epimerase. The value of $K_m, \text{ app}$ for UDP-GlcNAc was found to be 0.6 $\pm$ 0.06 mM. These values agree well with the $K_m, \text{ app}$ and $n_{\text{app}}$ values of 0.6 mM and 2.0, respectively, reported by Kawamura et al.$^{66}$ UDPE is a homodimeric enzyme with one substrate binding site per subunit. A $n_{\text{app}}$ of 1.8 thus indicates very strong cooperativity between the subunits. (Please refer to section 3.4, pg. 97 for an interpretation of the values of $n_{\text{app}}$).
Fig. 3-17 Plots of rate versus concentration of UDP-GlcNAc for WT, H213N and K15A UDPE.

The kinetic constants obtained for wild-type and mutant UDPE are given in Table 3-4 (pg. 127). The values were obtained by computer fitting the data to the Hill equation (equation 3.6).

\[
\frac{v}{V_{\text{max}}} = \frac{[S]^n}{K_{m,\text{app}} + [S]^n}
\]  

(3.6)

As will be further discussed in section 3.6.12 (pg. 115), our results point towards the allostery in UDPE as a V-system. For a V-type homotropic allosteric enzyme, the rate equation is given by equation 3.10.
\[
\frac{v}{V_{\text{max}}} = \frac{(2/\beta) [S]}{K_S} + \frac{[S]^2}{K_S^2} \frac{1}{1 + \frac{2[S]}{K_S} + \frac{[S]^2}{K_S^2}}
\] (3.10)

where \( V_{\text{max}} = \beta k_{\text{cat}} [E] \) (3.11)

\( \beta \) is the interaction factor affecting \( V_{\text{max}} \). Since we do not know the value of \( \beta \), and since the amount of enzyme used for rate measurements varies from mutant to mutant, for the purpose of comparison we have reported \( k_{\text{cat, app}} \).

\[
k_{\text{cat, app}} = \frac{V_{\text{max, app}}}{[E]}
\] (3.14)

Similarly, the \( K_{m, \text{app}} \) in the Hill equation (equation 3.6) is given by

\[
K_{m, \text{app}} = (a^{n-1}b^{n-2}c^{n-3} \ldots z^1) K_S^n = n\sqrt{[S]_{0.5}}
\]

Since UDPE is a \( V \)-system, the interaction factor for substrate binding, \( a, b, c, \) etc will be equal to 1. Thus,

\[
K_{m, \text{app}} = K_S^n
\] (3.15)

where \( K_S \) is the intrinsic binding constant for each site in UDPE. For the wild-type epimerase with a \( K_{m, \text{app}} \) of 0.6 mM and \( n \) value of 1.8, the intrinsic dissociation constant \( (K_S) \) is 0.75 mM.

Among the five mutants prepared, H213N was the only mutant that could be completely kinetically characterized. It showed about a 50-fold decrease in \( k_{\text{cat, app}} \), which was not significant enough to characterize His213 as a key catalytic residue involved in protonating the
leaving UDP. However, the possibility of His213 assisting the anomeric C-O bond cleavage to some degree cannot be completely ruled out. Since UDP is a good leaving group, a residue that assists the C-O bond cleavage might not be very critical and a mutation in that residue might not affect the value of $V_{\text{max}}$ considerably. Alternatively, the 50-fold decrease in $k_{\text{cat, app}}$ could be a result of the effect of mutation on cooperativity. The value of $n_{\text{app}}$ for this mutant shows a decrease in cooperativity (1.31 as opposed to 1.8 in WT epimerase). In general, in a $V$-system a decrease in cooperativity results in a decrease in $V_{\text{max}}$. This could be the case in the H213N mutant. The $K_{m, \text{app}}$ ($=K_s^o$) of H213N is 17.2 mM, which corresponds to an intrinsic dissociation constant ($K_s$) value of 8.8 mM. Assuming that UDPE is indeed a $V$-system, a 12-fold decrease in $K_s$ indicates that His213 residue is involved in substrate binding. This is also supported by the fact that this residue is H-bonded to the oxygen of the $\beta$-phosphate of UDP in the closed subunit.

With the K15A mutant, the $K_{m, \text{app}}$ was so high that saturation could not be reached even at 130 mM UDP-GlcNAc. A $>200$-fold increase in $K_{m, \text{app}}$ in K15A mutant suggests that a mutation of this residue has probably affected the intrinsic dissociation constant ($K_s$) of the enzyme. In both subunits, the Lys15 residue is positioned close to the phosphate of UDP. It is also found to form a salt-bridge with Glu117, suggesting that it is probably positively charged. Thus, Lys15 might be involved in binding the negative charge of the UDP portion of the substrate.

The three carboxylate mutants, D95N, E117Q and E131Q, could not be characterized kinetically as their activity was $<0.01\%$ of the WT epimerase. Some preliminary tests showed that the rate increased with substrate concentration up to about 1 mM and increases in substrate concentration beyond 3 mM did not considerably increase their activity. However, an increase
in enzyme concentration did result in an increase in rate. This suggests that the $K_{m, \text{app}}$ of these mutants is somewhere around 1 mM, very close to the $K_{m, \text{app}}$ of WT enzyme. Thus, these residues are probably not involved in substrate binding. The value of $k_{\text{cat, app}}$ given in Table 3-4, (pg. 127) was determined from the rate observed at 8 mM UDP-GlcNAc. All three of these carboxylate mutants showed a decrease of four orders of magnitude in the value of $k_{\text{cat, app}}$.

This large decrease in the value of $k_{\text{cat, app}}$ in these mutants could be a result of the mutation affecting catalysis and/or allostery. Another possibility considered early on in this project was that the small rate observed is due to a wild-type contamination in the enzyme preparation. To check this possibility the E117Q and WT epimerase were separately expressed as histidine-tagged enzymes.

3.7.5 Preparation and Characterization of the His-tagged Wild-Type and E117Q Mutant UDPE

In order to confirm that the small rate that we see with the E117Q mutant is indeed not a wild-type contamination, both the wild-type and E117Q mutant enzymes were expressed as histidine-tagged enzymes. A protein with a histidine-tag at its N- or C-terminus can be separated from untagged contaminants using affinity chromatography. The plasmids pKI86 and pJT03 were used to amplify the genes for the wild-type and E117Q mutant enzymes. From the DNA sequence of the regions immediately before and after the nfrC gene in pKI86 we were able to find one cut site before the gene but none after the gene. In the absence of a suitable cut-site it was not possible to use the traditional methods of sub-cloning using a ligation strategy. Consequently, we decided to use Novagen’s Ligation independent cloning (LIC) system and
subclone the genes for the WT and E117Q epimerases independently into pET-30 Xa/LIC vector.

The pET-30 Xa/LIC vector is designed so that it allows the directional cloning of PCR products without the need for restriction enzyme digestion or ligation reactions (Fig. 3-18, pg. 133). When the linearized LIC vectors are treated with T4 DNA polymerase in the presence of only one dNTP the 3' to 5' exonuclease activity of the polymerase removes nucleotides from the ends of the vector until it encounters a residue corresponding to the single dNTP present in the reaction mixture. This produces specific, non-complementary 12 to 14 base single stranded overhangs at the two ends of the vector. PCR products similarly treated and with specific complementary overhangs could then be annealed to the vector. The annealed LIC vector and insert are then transformed into competent *E. coli* cells, which form covalent bonds at the vector-insert junction to yield circular plasmids.
Immediately preceding the cloning site, the pET-30 Xa/LIC vector contains nucleotide sequences coding for an N-terminal histidine-tag (His-tag) and S-tag. As a result, when plasmids constructed from this vector are transformed into bacteria, they will overexpress proteins with an N-terminal His-tag and S-tag for purification and detection, respectively. Proteins containing the 6 amino acid histidine-tag can be easily purified by affinity chromatography using a nickel-bound column. The His-tag binds to the nickel on the column through chelation. After washing out all the untagged impurities, the pure tagged protein can be eluted with a gradient of imidazole buffer. Recombinant proteins that contain an S-tag can be detected and quantified.
using fluorescence and western blot techniques. Furthermore, the vectors are so designed that both the tags in the overexpressed protein can be removed with the protease Factor-Xa. Alternatively, thrombin could be used to selectively remove the His-tag. The blueprint of the vector near the cloning site is shown in Fig. 3-19 (pg. 134). The amino acid sequence added to the N-terminus of the recombinant protein is also shown.

A.

\[ \text{Thrombin cut site} \]
\[ \text{Xa/LIC site} \]

B.

\[ \text{His-tag} \]
\[ \text{S-tag} \]
\[ \text{MCS} \]
\[ \text{His-tag} \]

Fig. 3-19 pET-30 Xa/LIC cloning/expression region. (A) Blue-print of the region. (MCS = multiple cloning site) (B) Nucleotide and amino acid sequence of the region showing the two tags, cut-sites and ligation independent cloning (LIC) site.

Another important feature of the pET-30 Xa/LIC vector is that it encodes the gene for kanamycin resistance. The template plasmids, pKJ86 and pJT03, from which the WT and E117Q genes were subcloned codes for ampicillin resistance. This difference in antibiotic
resistance could be used as a selection criterion and avoid contamination of the colonies containing the subcloned plasmid with colonies containing the template pK186 plasmid.

The plasmids obtained by subcloning the WT and E117Q mutant genes into the pET-30 Xa/LIC vector were called pJS22 and pTB11, respectively. The cells containing the plasmids were grown in a culture containing kanamycin. When induced with IPTG, they overexpressed the respective proteins to about 30 – 40% of the total cell protein (Fig. 3-20A, pg. 135). The his-tagged WT (His-WT) and E117Q (His-E117Q) enzyme were purified by procedures mentioned in the experimental section to obtain >95% pure protein (Fig. 3-20B, pg. 135).

![Mol. Wt std](A) ![Mol. Wt std](B)

<table>
<thead>
<tr>
<th>Mol. Wt std</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>66kDa</td>
<td></td>
</tr>
<tr>
<td>30kDa</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3-20 SDS-PAGE gels showing the overexpression of His-E117Q (A, lane 1) and purified His-E117Q (B, lane 1).

The purified His-WT and His-E117Q enzymes were characterized by ESI-MS. The calculated mass of the WT UDPE is 42,244 Da. The calculated mass of the additional amino acids from cloning (shown in Fig. 3-19, pg. 134) is 4,986 Da. Thus, the calculated mass of His-
WT should be 47,230 Da. Both enzymes showed masses consistent with the calculated values (Table 3-5, pg. 136). When His-WT was checked for its ability to catalyze the epimerization of UDP-GlcNAc it was observed that the presence of the additional histidine-tag did not affect the activity. The His-E117Q also showed rates of forward reaction similar to that of the untagged E117Q mutant, suggesting that the slow rate observed with the E117Q mutant was indeed due to the mutant and not due to any WT contamination.

**Table 3-5. Mass of His-WT and His-E117Q**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Calculated mass (Da)</th>
<th>Observed mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>His-WT</td>
<td>47,230</td>
<td>47,226 ± 10</td>
</tr>
<tr>
<td>His-E117Q</td>
<td>47,229</td>
<td>47,226 ± 10</td>
</tr>
</tbody>
</table>

One of the problems commonly faced in purifying mutants from wild-type contamination by tagging and affinity chromatography is that sometimes one or more of the tagged mutant subunits exchanges with the untagged wild-type contaminant while the two are together inside the cell or during purification. This is likely not happening in our case since there appears to be a high association constant between the subunits. This was checked by measuring the specific activity of the WT epimerase at different enzyme concentrations. It was observed that the specific activity remains a constant in the range 1 μg/mL to 0.01 μg/mL. A similar observation was also made by Kawamura et al.66 In general, as the concentration of enzyme decreases, depending on the association constant between subunits, the amount of enzyme existing as a monomer increases. If we assume that the monomeric enzyme is inactive, then if there were increased dissociation at low enzyme concentrations, we should have seen a decrease in specific activity. The fact that the specific activity of the homodimeric UDPE remains a constant in the range 1μg/mL to 0.01 μg/mL suggests a high association constant.
between the subunits. Since the His-WT and His-E117Q showed activity similar to that of the untagged WT and E117Q epimerases, respectively, no further studies were done with the histidine-tagged enzymes.

### 3.7.6 Intermediate Release

The mutagenesis results implicate three residues as potential catalytic acid/base residues – Asp95, Glu117 and Glu131. Mutants of all three residues show four orders of magnitude decrease in $k_{\text{cat}, \text{app}}$. An alternate explanation that could hold for an enzyme that exhibits cooperativity in catalytic efficiency ($V$-system) is that the decrease in $k_{\text{cat}, \text{app}}$ observed could be a result of effect of the mutation on allostery. One way to differentiate between the effect on catalysis and allostery would be to look for the ability of the mutants to form the intermediates. All epimerases that employ a deprotonation/reprotonation step operate by a "two-base" mechanism. This means two separate bases are involved in deprotonating the two epimers. Suppose $B_1$ and $B_2$ are the bases involved in deprotonating UDP-GlcNAc and UDP-ManNAc, respectively (Fig. 3-21, pg. 138). If we mutate $B_1$ and assay the reaction in the forward direction (UDP-GlcNAc → UDP-ManNAc), the enzyme would be catalytically severely impaired and would not be able to produce either the product, UDP-ManNAc, or the intermediates. Likewise, if we mutate $B_2$ and assay the reaction in the forward direction, we would still see a severely catalytically impaired mutant with respect to product formation. However, this mutant might still be able to convert UDP-GlcNAc into the intermediates. Thus, by monitoring the ability of these mutants to produce the intermediates, one could learn about the roles of these residues in catalysis.
Fig. 3-21 Effect of mutation of either of the two catalytic residues.

From the literature we know that WT epimerase releases the intermediates, UDP and glycal, upon extended incubation with its substrates. The intermediate release for both the WT and the mutant enzymes was monitored by HPLC. A UDP-GlcNAc concentration of 8 mM was used for all intermediate release assays. At this concentration the WT, D95N, E117Q and E131Q epimerases are saturated while H213N and K15A are sub-saturated. On incubating the WT and mutant enzymes with UDP-GlcNAc for extended periods of time, it was observed that all mutants except E131Q and D95N showed intermediate release (data not shown).
A coupled assay was later used to determine a more accurate rate of UDP release (Fig. 3-22, pg. 139). The assay is based on the fact that although pyruvate kinase normally uses ADP as its substrate, it is also able to use UDP as an alternate substrate. Pyruvate kinase catalyzes the transfer of a phosphate group from phosphoenol pyruvate (PEP) to UDP to give pyruvate and UTP (uridine triphosphate). The pyruvate is then reduced by lactate dehydrogenase (LDH) with simultaneous oxidation of NADH. The rate of release of UDP is measured as a function of the rate of consumption of NADH, and determined by following the decrease in absorbance at 340 nm at 37 °C. Since UDP is not its natural substrate, large quantities of the enzyme must be used in the assay. Interestingly, both the stopped and coupled assays showed that all three mutants, H213N, K15A and E117Q, released intermediates at almost the same rate as product formation (epimerization). With the WT enzyme, the rate of intermediate release was found to be almost 1000 times slower than the rate of epimerization. In the case of H213N and K15A, the rates shown in Table 3-6 (pg. 140) are under sub-saturation conditions. The His213 and Lys15 residues are involved in substrate binding and mutants of these residues have high values of $K_{\text{m, app}}$. The fact that these mutants release the intermediates at rates comparable to that of epimerization is not surprising since the affinity for UDP has likely been reduced. The E117Q mutant, however, shows a 10,000-fold decrease in the magnitude of $k_{\text{cat}}$ but is able to form the intermediates from UDP-GlcNAc at rates comparable to that of the WT epimerase. This would be consistent with its role as the base $B_2$ involved in deprotonating UDP-ManNAc (Fig. 3-21,
An alternate explanation could be that the low $k_{\text{cat}}$ is a result of impaired allostery in this mutant and the low rate of epimerization/UDP release is due to an increased rate of active site "opening". Further experiments described in the following sections indicate that the allostery has been affected in this mutant.

Table 3-6. Comparison of activity of epimerization and intermediate release in the presence of 8 mM UDP-GlcNAc

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_{m,\text{app}}$ (mM)</th>
<th>Epimerization (μmoles/min/mg)</th>
<th>UDP release (μmoles/min/mg)</th>
<th>Epimerization/UDP release ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.6</td>
<td>7.6</td>
<td>$1.0 \times 10^{-2}$</td>
<td>$1.5 \times 10^{3}$</td>
</tr>
<tr>
<td>E117Q</td>
<td>-</td>
<td>$0.6 \times 10^{-3}$</td>
<td>$1.0 \times 10^{-3}$</td>
<td>1</td>
</tr>
<tr>
<td>D95N</td>
<td>-</td>
<td>$0.5 \times 10^{-3}$</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E131Q</td>
<td>-</td>
<td>$0.6 \times 10^{-3}$</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>H213N</td>
<td>17.2</td>
<td>$7.9 \times 10^{-2}$</td>
<td>$9.0 \times 10^{-3}$</td>
<td>9</td>
</tr>
<tr>
<td>K15A</td>
<td>&gt;100</td>
<td>$4.5 \times 10^{-3}$</td>
<td>$2.4 \times 10^{-3}$</td>
<td>2</td>
</tr>
</tbody>
</table>

All the above data have an error margin of about 10%.

Attempts to follow the rate of intermediate release with UDP-ManNAc as the substrate, to identify the base $B_1$ were hampered by the fact that UDP-ManNAc tends to undergo slow spontaneous dissociation into UDP and sugar when incubated at pH 8.8 and 37 °C. This rate of spontaneous dissociation (as determined with a control sample) was comparable to the rate of UDP release observed with UDP-GlcNAc as substrate.
One of the original questions we had with the three carboxylate mutants, D95N, E117Q and E131Q, was whether the small (and similar) rate observed with the three mutants was due to wild-type contamination in the mutant protein preparations. However, if this was the case, since the rate of epimerization is 1000-fold slower than the rate of UDP release in the WT enzyme; we should not have observed any intermediate release with the mutant enzyme preparations. The fact that E117Q catalyzes intermediate release at the same rate as epimerization proves that the small rate of epimerization seen with this mutant is not due to wild-type contamination.

3.7.7 Inhibition of UDPE

The intermediate, UDP, is also known to inhibit the WT epimerase with a $K_i$ value of 1 mM.66 Thus, with the wild-type epimerase, as the intermediates accumulate, the enzyme is gradually inhibited. While carrying out the stopped assay for intermediate release in H213N, it was observed that this mutant was not inhibited by UDP. A preliminary study with the coupled epimerization assay showed that very little inhibition is observed with the H213N mutant even at 10 mM UDP. Kawamura et al. showed that in the WT epimerase, UDP does not affect the $K_m$, $a_{pp}$ for UDP-GlcNAc but affects its $V_{max}$.66 In other words, it is a $V$-type (non-competitive) inhibitor (please refer to section 2.3, pg. 41 for an explanation of non-competitive inhibition). Evidence presented in this chapter further confirms that UDPE is a $V$-system. In such systems an inhibitor that binds to the regulatory site would behave as a non-competitive inhibitor. From kinetic studies we know that the H213N mutation has partially impaired the allosteric control of the enzyme (lower value of $n_{app}$). In other words, the binding of UDP-GlcNAc to the regulatory site does not considerably change the catalytic efficiency of the active site. Under these conditions an inhibitor that binds to the regulatory site would be less effective. Furthermore, in the X-ray crystal structure the His213 residue has been found to be H-bonded to the oxygen of
the β-phosphate of UDP, and kinetic studies also show that this residue plays an important role in binding. Considering these facts, it is not surprising that the H213N mutant has a lower affinity for the UDP inhibitor.

**Fig. 3-23 Structures of molecules tested as inhibitors of UDPE**

A series of other molecules were tested as mechanism based inhibitors of UDPE. These include N-acetylglucosamine thiazoline or NAG-thiazoline (4), 1-deoxynojirimycin (5), 2-acetamido-1,2-dideoxynojirimycin (6) and 2-acetamido-2-deoxy-D-glucono-1,5-lactone (7). UDPE catalyzes the epimerization reaction via an anomeric C-O bond cleavage resulting in the formation of UDP and glycal as intermediates (Fig. 3-1, pg. 81). This is an anti-elimination in the case of UDP-GlcNAc and a syn-elimination in the case of UDP-ManNAc. Due to the high pKₐ of the C-2 proton, an E1cb mechanism can be ruled out. Alternatively, an E2 or E1 mechanism could be at play. An E2 syn-elimination is generally unfavorable due to the eclipsed high energy transition state.

In UDP-ManNAc, an E1 mechanism of UDP elimination could be aided by anchimeric assistance from the N-acetyl group. Neighboring group participation by the N-acetyl group would lead to release of UDP and an initial oxazoline intermediate which then undergoes deprotonation at C-2 and ring opening to give the glycal intermediate. NAG-thiazoline (4)
would mimic this oxazoline intermediate. It was obtained from Dr. Spencer Knapp and Dr. Steve Withers, who previously demonstrated that it was a potent inhibitor of N-acetylhexosaminidases. These are glycosidases that catalyze the anomeric C-O bond cleavage of N-acetylhexosamines in glycolipids and glycoproteins. Studies with NAG-thiazoline as an inhibitor strongly indicated that the initial anomeric C-O bond cleavage is assisted by neighboring group participation by the 2-acetamido group in N-acetylhexosaminidases. An E1 mechanism involving elimination of UDP would give a positively charged oxocarbenium-like transition-state. This positive charge would be distributed between the ring oxygen and C1. Nojirimycin derivatives 5 and 6 which mimic this positively charged transition-state are also known to be good inhibitors of glycosidases. But unfortunately, no inhibition of the UDPE reaction was observed with compounds 4 to 6 (1 mM) in the presence or absence of added UDP (1 mM).

D-Gluconolactone (7) resembles the oxocarbenium and glycal intermediates through its flattened chair geometry and its partial charge separation. Gluconolactones are also known inhibitors of glycosidases and glycogen phosphorylase, both of which are known to stabilize oxocarbenium ion-like transition states. Again, no inhibition by 5 at 1 mM was found in UDPE in the presence or absence of UDP (1 mM). It could be that major binding energy for the substrates comes from the UDP portion of the sugar nucleotide. This has been observed in other enzymes that use sugar nucleotides as substrates. In the case of UDPE, the value of $K_{m, app}$ for UDP-GlcNAc is 0.6 mM, while the $K_i$ for UDP is 1 mM. It is possible that since none of the inhibitors we tried were in the sugar nucleotide form, they were not able to bind to the enzyme and hence did not show any inhibition. Furthermore, the transition state for UDPE may not be similar to that of glycosidases. The leaving group in glycosidases is an alkoxide as
opposed to a phosphate in UDPE. The transition state for UDPE is likely similar to that of a transferase, for which very few inhibitors are known.

3.7.8 Synthesis of UDP-ManNAc

In order to study the epimerization of UDP-ManNAc by UDPE, the substrate had to be synthesized. This could be done by either enzymatic or chemical methods. Two enzymatic syntheses of UDP-ManNAc are given in the literature and both start with UDP-GlcNAc. After the enzymatic synthesis, the UDP-ManNAc needs to be separated from residual UDP-GlcNAc using conventional chromatographic techniques. In the past when other groups used UDP-ManNAc prepared by this approach as a substrate for studying UDPE, it was observed that the small contamination of UDP-GlcNAc in UDP-ManNAc preparations could autocatalyze the epimerization reaction. This is because, in addition to being the substrate, UDP-GlcNAc is also the allosteric activator of UDPE. For this reason we decided to synthesize UDP-ManNAc chemically.

Two groups, Salo and Fletcher and Yamazaki et al., have reported the syntheses of UDP-ManNAc previously. They both used the oxazoline derivative of ManNAc to prepare ManNAc α-1-phosphate, which was then coupled to UMP derivatives. Here, we have used a similar approach; however, the method for obtaining the oxazoline and α-1-phosphate derivatives of ManNAc was modified (Fig. 3-24, pg. 145). When we tried the acetyl chloride method for preparing the oxazoline derivative (2) used by Yamazaki et al., a dark brown/purple product was obtained which was hard to purify. We, therefore, used an alternative approach using simple conventional techniques. ManNAc was first protected by peracetylation using an acetic anhydride/pyridine mixture. The anomeric position was then brominated using HBr in
acetic acid and the product (1) was purified on an alumina (neutral) column. This reaction was much cleaner and faster and gave a fairly good yield (35%). Conversion of (1) into the oxazoline derivative (2) using K₂CO₃ was almost quantitative and could be easily obtained as pure crystals from ethyl acetate/hexane with a yield of 90%.

![Synthesis of UDP-ManNAc](image)

**Fig. 3-24 Synthesis of UDP-ManNAc.** (DBP = dibenzyl phosphate and DCE = dichloroethane)

The synthesis of peracetylated ManNAc α-1-phosphate was also slightly modified from that used by Yamazaki et al. The coupling of the oxazoline derivative (2) and dibenzyl phosphate was done in 1,2-dichloroethane for 1 hr under a dry, inert atmosphere. The reaction mixture was immediately diluted with methanol and hydrogenated to give the peracetylated
ManNAc $\alpha$-1-phosphate. The product was purified using preparative TLC and then deacetylated with NaOMe/MeOH to give pure ManNAc $\alpha$-1-phosphate (3). Synthesis of ManNAc $\alpha$-1-phosphate from the oxazoline gives stereoselectively the $\alpha$-product under both kinetic and thermodynamic conditions. This makes it a very attractive route for the synthesis of $\alpha$-derivatives of ManNAc. ManNAc $\alpha$-1-phosphate was coupled to UMP-morpholidate using the modified Khorana-Moffat method used by Salo and Fletcher to give UDP-ManNAc in 12% yield. The 400 MHz proton NMR spectra of compounds 1 to 3 and UDP-ManNAc are shown below. The peaks were assigned using 2-D proton NMR (COSY).
3.7.9 Kinetics of the Reverse Reaction

In an attempt to fully understand the roles of the mutated residues in catalysis and/or allostery, we wished to monitor the ability of the mutants to catalyze the reaction in both directions. Work done by Kawamura et al. showed that the WT epimerase have an absolute requirement for UDP-GlcNAc in catalyzing the epimerization of UDP-ManNAc (reverse reaction). When pure UDP-ManNAc was used as a substrate for the WT epimerase, no reaction was observed even with a 15-fold increase in enzyme concentration. The concentration of UDP-GlcNAc required to achieve half maximal activation of the reverse reaction was estimated to be 0.6 mM (equal to $K_{m, \text{app}}$ of UDP-GlcNAc). Morgan et al. also observed this tight regulation in the epimerase. When they incubated the enzyme with 1.1 mM UDP-ManNAc in the presence or absence of 0.35 mM UDP-GlcNAc for 10 min, about 28% conversion was
observed in the sample containing the activator. However, no epimerization was observed in the sample lacking the activator even after 120 min.

Fig. 3-25 HPLC chromatograms for the WT epimerase. (A). Enzyme incubated with UDP-GlcNAc after 10 min. (B). Enzyme incubated with UDP-ManNAc after 24 hrs.

We further investigated the extent of regulation in the WT epimerase by changing both the period of incubation and the amount of enzyme. A stopped assay using HPLC was used to compare the forward and reverse reactions. With 0.01 units of the WT epimerase and 1 mM UDP-GlcNAc, conversion of 50% to equilibrium (at equilibrium UDP-GlcNAc : UDP-ManNAc = 10 : 1) was observed in 10 min. However, with the same amount of enzyme and UDP-ManNAc as the substrate, <1% epimerization was observed even after 24 hrs (Fig. 3-25, pg. 149). When the amount of enzyme was increased to 0.45 units, equilibrium was reached within 5 min even with pure UDP-ManNAc. An attempt at determining the rate of the unactivated reverse reaction failed because the rates of the forward reaction and the activated reverse reaction are so much faster than the rate of the unactivated reverse reaction in the WT epimerase. As a result, at an enzyme concentration that is required to see any significant rate in
the reverse direction, as soon as the first turnover of UDP-ManNAc into UDP-GlcNAc occurs, the reaction is autocatalyzed and equilibrium is reached instantly.

Fig. 3-26 HPLC chromatograms for E117Q mutant epimerase. (A). Enzyme incubated with UDP-GlcNAc after 2 hrs. (B). Enzyme incubated with UDP-ManNAc after 12 hrs.

These results suggest that UDPE exists in two different conformations – an active conformation and an inactive conformation. In the absence of UDP-GlcNAc (the activator) the epimerase is in the inactive form. Binding of UDP-GlcNAc to one subunit induces a conformational change that converts the enzyme into the active form. At high enzyme concentrations only a very small percentage of the protein is in the active conformation. This small percentage is enough to bring about the first few turnovers of UDP-ManNAc to UDP-GlcNAc, which can then activate further enzyme molecules. This autocatalytic process is
probably responsible for the instant equilibration observed with UDP-ManNAc when the enzyme concentration was increased.

Since the D95N, E117Q and E131Q mutants are much poorer catalysts, it was possible to follow the forward and the reverse reaction with 1 mg/mL (6.0 x 10^4 units) enzyme and 1 mM substrate. As observed with the continuous assay, the rate of the forward reaction was almost the same for all three mutants. When these mutants were incubated with 1 mM UDP-GlcNAc, about 2 -3 % conversion to equilibrium was observed in 2 hours (Fig. 3-26, pg. 150). To determine the rate of unactivated epimerization of UDP-ManNAc we did not have an enzyme that could continuously remove the product and activator, UDP-GlcNAc. Previous studies show that UDPE requires about 0.5 mM UDP-GlcNAc to achieve half-maximal activity in the reverse direction. At about 0.02 mM UDP-GlcNAc (2-3% of equilibrium concentration), the reverse reaction will still be greatly unactivated. When 1 mM UDP-ManNAc was incubated with E117Q, D95N and E131Q, 2-3 % conversion to equilibrium was observed in 9 - 12 hrs. Comparing the time required to achieve 2 -3% conversion to equilibrium in either direction we see that in all three mutants there is still some degree of cooperativity, although it is greatly compromised as compared to that of the WT epimerase. The fact that D95N, E117Q and E131Q behave differently from the WT epimerase in a comparison of the forward and reverse reactions shows that the observed slow rates of epimerization are due to the mutants and not due to a wild-type contamination in the enzyme preparation.
3.7.10 Fluorescence Studies

Fig. 3-27 Position of the four tryptophan residues (shown in green) in the UDPE dimer

As mentioned in previous sections of this chapter, a complete interpretation of the results from kinetic studies on UDPE mutants is not possible without understanding the mechanism of allostery in this enzyme. However, the techniques that can be used to study the allostery in UDPE are restricted by the fact that the substrate of this enzyme is also the effector. An attempt was made to probe the substrate/effector binding in UDPE using fluorescence spectroscopy. The tryptophan, tyrosine and phenylalanine residues in proteins absorb at 280, 274 and 257 nm and fluoresce at 340, 303 and 282 nm, respectively. Of the three, tryptophan has the highest fluorescence quantum yield and is very sensitive to its environment. This makes it a good intrinsic probe to study conformational changes and interactions within proteins. In general, the
intensity, quantum yield, and wavelength of maximum fluorescence emission of tryptophan residues are very solvent dependent. The fluorescence spectrum shifts to shorter wavelength and the intensity of the fluorescence increases as the polarity of the solvent surrounding the tryptophan residue decreases. Thus tryptophan residues which are buried in the hydrophobic core of proteins can exhibit emission maxima that are shifted by 10 to 20 nm compared to tryptophan residues on the surface of the protein. Any event that changes the conformation of the protein and in turn changes the environment of a tryptophan residue can thus be followed by measuring the change in wavelength and/or intensity of tryptophan fluorescence. The wild-type epimerase has four tryptophan residues (Fig. 3-27, pg. 152). A fluorescence scan of the epimerase showed that the enzyme exhibits strong tryptophan fluorescence at 335 nm (excitation 280 nm) (Fig. 3-15, pg. 126). A look at the structure of UDPE shows that one of the four tryptophan residues (Trp129) is located at the dimer interface and one at the domain interface in each subunit (Trp179). The other two tryptophans (Trp278 and Trp284) are located within the C-terminal domain that contains the Rossmann dinucleotide binding fold. Due to the critical positioning of these tryptophan residues, binding of a ligand at the nucleotide binding region and any conformational change across the dimer interface resulting from ligand binding may result in a change in fluorescence.

For the binding studies with UDPE, there were certain limitations that had to be considered while using the fluorescence technique. The UDP portion of the substrates has a strong absorption at 260 nm. Even at 280 nm the absorbance is strong enough to cause a filtering effect (decrease in intensity of the incident light as it travels from one end of the cuvette to the other end). To avoid this, the excitation wavelength was moved to 295 nm and the concentration of the substrates was always kept below 2.5 mM. The concentration of enzyme for further experiments was chosen to be 0.5 mg/mL. At this concentration, there is a linear
relationship between the intensity of fluorescence and enzyme concentration (Fig. 3-28, pg. 154). Under these conditions the wild-type epimerase shows a 50% increase in tryptophan fluorescence intensity on addition of 1 mM UDP-GlcNAc. This change in fluorescence could be due to the binding event or due to the conformational change related to the allosteric control of the enzyme.

A problem with the interpretation of the fluorescence data, however, is that with the wild-type epimerase at 0.5 mg/mL, it is not possible to determine the change in fluorescence upon binding of UDP-GlcNAc and UDP-ManNAc separately. This is because at this concentration epimerization of the substrate is very fast and equilibrium (UDP-GlcNAc / UDP-ManNAc = 10:1) is reached instantly. Kinetic studies show that in all three carboxylate mutants (E117Q, D95N and E131Q), however, the value of $V_{\text{max, app}}$ has been reduced by about 4 orders of magnitude compared to that of the wild-type epimerase. This allows us to measure the change in fluorescence upon binding of pure UDP-GlcNAc and pure UDP-ManNAc with these mutants. The $K_{m, \text{app}}$ of these mutants is known to be close to that of the WT enzyme (0.6 mM) which would ensure that there will be saturation binding of the ligands at concentrations of 2.0 mM.

![Fig. 3-28 Relationship between intensity of fluorescence and enzyme concentration](image-url)
However, a comparison of the forward and reverse reactions catalyzed by these mutants shows that they have compromised allosteric regulation as compared to that of the wild-type epimerase. If the change in fluorescence we observed with the wild-type epimerase was indeed due to a conformational change following the binding of UDP-GlcNAc to the regulatory site, then these mutants should show either no change in fluorescence or a change in fluorescence that is different from that of the WT epimerase upon the addition of UDP-GlcNAc.

Table 3-7 Percentage change in fluorescence in wild-type and mutant epimerases with ligand binding.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>E117Q</th>
<th>D95N</th>
<th>E131Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-GlcNAc (1 mM)</td>
<td>50 ± 2*</td>
<td>110 ± 10</td>
<td>9 ± 1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>UDP-ManNAc (1 mM)</td>
<td>-</td>
<td>9 ± 1</td>
<td>9 ± 1</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

* In the cuvette containing wild-type epimerase and UDP-GlcNAc there is an equilibrium mixture of UDP-GlcNAc and UDP-ManNAc.

With the D95N and E131Q mutants about 9% increase in fluorescence was observed in the presence of either 1 mM UDP-GlcNAc or 1 mM UDP-ManNAc (Table 3-7, pg. 155). From kinetic studies we know that at this concentration UDP-GlcNAc binds to these mutants, although we are not at saturation. The observation of the same amount of fluorescence increase with pure UDP-ManNAc suggests that it also binds to the enzyme with similar affinity even in the absence of the activator. Since the increase in fluorescence was small even with approximately $K_m,_{app}$ concentrations of UDP-GlcNAc, it was not possible to determine the stoichiometry of binding. With the E117Q mutant, again, a 9% increase in fluorescence was
observed with 1 mM UDP-ManNAc, suggesting that it is also able to bind UDP-ManNAc in the absence of UDP-GlcNAc. The wild-type epimerase showed the same 9% increase in fluorescence with 1 mM UDP ($K_i = 1$ mM). These observations suggest that the 9% increase in fluorescence is related to binding of the nucleotide in the Rossmann fold. The fact that the enzyme is capable of binding UDP-ManNAc in the absence of UDP-GlcNAc argues against cooperativity in substrate binding. In other words, the ability of the mutants to bind UDP-ManNAc and UDP-GlcNAc to the same extent suggests that UDPE is a $V$-system (please refer to section 3.3, pg. 92 for a definition of $V$-system).

The E117Q mutant showed about 110% increase in fluorescence with 1 mM UDP-GlcNAc. As mentioned earlier, the wild-type epimerase showed a 50% increase in fluorescence with 0.9 mM UDP-GlcNAc and 0.09 mM UDP-ManNAc (equilibrium mixture). These observations suggest that the major change in fluorescence observed with the WT enzyme and the E117Q mutant is probably due to the conformational change following the binding of UDP-GlcNAc to the regulatory site. Interestingly, the change in fluorescence with UDP-GlcNAc in E117Q occurred slowly and took about 5 min to reach the maximum (Fig. 3-29, pg. 157). This could be because the binding event itself is slow, or the rate of conformational change has been reduced by mutation. The fact that such a slow change in fluorescence is not observed in this mutant with UDP-ManNAc suggests that the slow change in fluorescence observed with UDP-GlcNAc results from a slow conformational change following the binding of UDP-GlcNAc to the regulatory site. In any event, the final conformation attained by the E117Q mutant is likely different from that of the active conformation of the WT epimerase (indicated by a 110% increase of fluorescence in the E117Q mutant as opposed to a 50% increase in the WT epimerase), resulting in the observed reduced cooperativity.
The observation that the D95N, E131Q and E117Q mutants show unusual fluorescence behavior suggests that they are unable to access the active conformation following the binding of UDP-GlcNAc to the regulatory site. This is likely responsible for the decreased allostery observed in these mutants. Furthermore, the fact that all three of these mutants show binding with 1 mM UDP-ManNAc suggests that UDPE is a V-system (Please refer to section 3.3, pg. 92 for a definition of V-system).

As discussed earlier, with the wild-type epimerase at 0.5 mg/mL it was not possible to measure the increase in fluorescence due to UDP-GlcNAc and UDP-ManNAc separately. Studies with the mutants suggest that UDPE is a V-system and the binding of the ligand (UDP-GlcNAc or UDP-ManNAc or UDP) to the two binding sites causes a 9% increase in fluorescence. The remaining 41% increase in fluorescence observed with the WT enzyme in the presence of 0.91 mM UDP-GlcNAc and 0.09 mM UDP-ManNAc (equilibrium mixture) is probably caused by the conformational change following binding of UDP-GlcNAc to the
regulatory site (Fig. 3-30, pg. 158). If this is true, then with a Scatchard plot we should obtain a stoichiometry of one ligand per dimer.

Fig. 3-30 A schematic representation of the various events following the addition of an equilibrated mixture of 0.91 mM UDP-GlcNAc and 0.09 mM UDP-ManNAc to wild-type UDPE. Squares represent the inactive conformation, circles represent the active catalytic subunit and the ellipse represent the regulatory subunit. The conversion of the inactive conformation into the active conformation (event shown in the dotted-box) is responsible for a 41% increase in fluorescence. All other events together are responsible for the 9% increase in fluorescence.

The increase in fluorescence at varying concentrations of UDP-GlcNAc was determined using 0.5 mg/mL wild-type epimerase. Corrections were made in the calculation of the substrate concentration to account for the fact that the actual concentration of UDP-GlcNAc in the cuvette is only 91% of that added due to epimerization. A direct plot of the % increase in fluorescence versus substrate concentration gave a hyperbolic curve with a dissociation constant ($K_s$) value of 0.6 mM (Fig. 3-31A, pg. 160). This value is comparable with the calculated value of $K_s$ of 0.75 mM obtained for UDP-GlcNAc by kinetic measurements. A Scatchard plot (equation 3.13) of the data (For a description of the Scatchard analysis please refer to the experimental section
3.6.12, pg. 115) was linear, indicating that there is primarily one type of binding site associated with the change in fluorescence (Fig. 3-3B, pg. 160). Furthermore, the number of binding sites determined from the Scatchard plot was 0.7. The fact that the value for the number of binding sites and the dissociation constant obtained for the WT epimerase was lower than the expected value (1 and 0.75 mM, respectively) could be due to errors introduced into the measurement by various factors like the filtering effect at higher substrate concentration and the small change (9%) in fluorescence caused by binding at the active site. Another factor contributing to the lower values of dissociation constant (1/k) and number of binding sites (n) could be introduced by the value of “L” in equation 3.13. “L” is the concentration of “free UDP-GlcNAc”. When the enzyme concentration is much lower than the ligand concentration, the concentration of the free ligand will be close to the concentration of ligand added. In our assays we have 5.75 μM enzyme and the fluorescence change is being measured in the range of 0.01 – 1.8 mM [UDP-GlcNAc]. Under these conditions, the concentration of free UDP-GlcNAc is not equal to the concentration of UDP-GlcNAc added, especially at the lower end of the concentration range studied, resulting in large errors in the B/L values at lower L values. We were also limited by the fact that measurements could not be done at concentrations above 1.8 mM due to filtering effect caused by the substrates.

\[
\frac{B}{L} = nk - kB
\]  

(3.13)
Fig. 3-31 A) Binding curve for UDP-GlcNAc with wild-type epimerase.

B) Scatchard plot

3.8 Conclusions

The catalytic and allosteric mechanism of UDPE was studied by a combination of site-directed mutagenesis, kinetics and fluorescence. On the basis of the crystal structure and sequence homology, five residues in UDPE were selected for mutagenesis studies. These residues were initially selected as potential catalytic acid/base residues. His213 and Asp95 were mutated to Asn, Glu131 and Glu117 were mutated to Gln, and Lys15 was mutated to Ala. All mutants were characterized by ESI-MS, CD and fluorescence. All showed CD spectra similar to that of the WT epimerase except for the E117Q mutant, which showed a small decrease in molar ellipticity. UDPE is a homodimeric enzyme and the mutants showed a higher tendency to dimerize through intersubunit disulfide linkages. They also showed small changes in the intensity of their tryptophan fluorescence spectra at 335 nm (excitation at 295 nm). UDPE has four tryptophan residues, one of them being at the dimer interface. The dimerization behavior,
CD spectra and the fluorescence spectra indicate that although the subunits are folded in the mutants, the mutations might have caused small changes in the conformation of the dimer.

The interpretation of kinetic data obtained from the mutants was complicated by the fact that UDPE is a homodimeric allosteric enzyme. It is regulated by one of its substrates, UDP-GlcNAc, and the rate versus [UDP-GlcNAc] curve is sigmoidal with a $n_{app}$ value of 1.8. For the reaction in the reverse direction, (UDP-ManNAc → UDP-GlcNAc) the enzyme exhibits an absolute requirement for UDP-GlcNAc. Previous work has shown that half-maximal activity for the epimerization of UDP-ManNAc is observed in the presence of 0.6 mM UDP-GlcNAc. Work mentioned in this chapter (Kinetics of the Reverse Reaction, section 3.7.9, pg. 148) shows that the allosteric regulation in UDPE is quite tight. The WT epimerase crystallized in the presence of UDP-GlcNAc shows two different conformations for the two subunits of the dimer (Fig. 1-13, section 1.3.2.2b, pg. 22) with a single binding site per subunit. The mechanism proposed for the allosteric regulation in UDPE is that binding of UDP-GlcNAc to one of the subunits induces a conformational change in the dimer, increasing the substrate affinity and/or the catalytic efficiency of the other subunit. Thus, in the UDPE dimer one of the subunit is regulatory in nature and the other is catalytic in nature. However, in the absence of information regarding whether the cooperativity in UDPE is in substrate binding ($K$-system) or in catalytic efficiency ($V$-system), interpretation of kinetic data is not possible.

Kinetic studies with the three carboxylate mutants, E117Q, D95N and E131Q, showed that they have been severely catalytically impaired but their affinity for the substrate and effector, UDP-GlcNAc, is similar to that of the WT epimerase. A comparison of the ability of these mutants to catalyze the epimerization of UDP-GlcNAc and unactivated epimerization of UDP-ManNAc shows that the allosteric regulation in these mutants has also been considerably compromised. If UDPE were a $K$-system, a mutation that resulted in a decrease in substrate
binding cooperativity would have resulted in an increase in its apparent dissociation constant \( (K_{m, \text{app}}) \). The fact that these mutants show a \( 10^4 \) fold decrease in the value of \( k_{\text{cat, app}} \) and their \( K_{m, \text{app}} \) remains nearly unchanged suggests that UDPE is a \( V \)-system.

Previously, Kawamura et al. have shown that the activated enzyme has similar \( K_{m, \text{app}} \) values for UDP-ManNAc and UDP-GlcNAc (0.5 mM and 0.6 mM, respectively). The fact that both E131Q and D95N show the same 9% increase in fluorescence with 1 mM UDP-GlcNAc and 1 mM UDP-ManNAc suggests that these mutants do bind UDP-ManNAc in the absence of UDP-GlcNAc, and the affinity of the unactivated enzyme for UDP-ManNAc is similar to the affinity of the activated enzyme for UDP-GlcNAc. Thus, the difference in rate of epimerization by activated and unactivated enzyme is mainly due to a difference in their catalytic efficiency (\( V \)-system).

The same 9% increase in fluorescence is also observed with E117Q in the presence of UDP-ManNAc but a 110% increase in fluorescence is observed with UDP-GlcNAc. This compares with a 50% increase in fluorescence when the wild-type enzyme is incubated with an equilibrium mixture of UDP-GlcNAc and UDP-ManNAc (UDP-GlcNAc / UDP-ManNAc : 10/1). The wild-type and the mutant enzymes also show a 9% increase in fluorescence with 1 mM UDP. The fact that all three mutants show a 9% increase in fluorescence on binding of UDP-ManNAc inspite of a difference in their intrinsic fluorescence in the absence of substrates (Fig. 3-15, pg. 126) suggests that this increase in fluorescence is probably associated with binding of the nucleotide within the Rossmann fold. The large increases in fluorescence (50% and 110%, respectively) in both the WT epimerase and the E117Q mutant with UDP-GlcNAc is probably associated with binding of UDP-GlcNAc to the regulatory site, followed by a conformational change across the dimer interface.
Previous work by Kawamura et al. showed that UDP is a \( V \)-type (non-competitive, section 2.3, pg. 41) inhibitor of UDPE. This can be understood if UDP binds to the regulatory subunit and affects the catalytic efficiency but not the binding in the active subunit. On the basis of these pieces of evidence we propose that UDPE is a \( V \)-system. Thus, binding of UDP-GlcNAc to one subunit of UDPE induces a conformational change across the dimer interface that makes the other subunit catalytically more efficient. Considering the fact that the value of \( n_{\text{app}} \) for UDPE is close to the number of binding sites for UDP-GlcNAc, it follows that the free enzyme is catalytically quite inactive.

The E117Q mutant shows a \( 10^4 \) fold decrease in \( k_{\text{cat, app}} \) for the epimerization reaction and release of intermediates from UDP-GlcNAc at a rate comparable to that of the wild-type epimerase. This suggests that E117Q might be the catalytic residue responsible for the protonation of 2-acetamidoglucal to give UDP-ManNAc. In the crystal structure the Glu117 residue forms a salt bridge with the Lys15 residue in the closed subunit. This means that Glu117 is in the deprotonated form and is capable of functioning as the catalytic residue. It is impossible, however, to detach the direct effects on catalysis from those on allostery and several lines of evidence indicate that the E117Q mutation has affected the conformation of the dimer. These include a slightly lower molar elipticity in the range of 190 – 240 nm, an intrinsic fluorescence about 16% lower than the wild-type epimerase, and an unusually slow conformational change on binding of UDP-GlcNAc. It is conceivable that the decrease in \( k_{\text{cat}} \) is due to the crippled allostery and the intermediate release is simply the result of a “sloppy” active-site. Similarly, it is likely that E131Q and D95N also play a role in catalysis, but due to the effect of mutation on their allosteric regulation and hence, catalytic efficiency, we could not reach at any solid conclusions regarding their role in catalysis. The His213 and Lys15 residues are involved in substrate binding and the His213 residue probably also plays a role in allosteric
regulation as indicated by the lower value of $n_{\text{app}}$. With a better understanding of the nature of allosteric regulation in UDPE it would now be easier to plan further mutagenesis experiments and interpret the kinetic results.
Appendix-1

Bacterial Cell Envelope

The cell envelope may be defined as the cell membrane and cell wall plus an outer membrane if one is present. The cell membrane is the area of the cell immediately surrounding the cytoplasm and is perhaps the most conserved structure in living cells. It is the major barrier in the cell, separating the inside of the cell from the outside. It is this structure which allows cells to selectively interact with their environment. The cell membrane essentially consists of phospholipids and membrane proteins (Fig. A-1.1, pg. 166). Phospholipids contain one or more charged or polar group (including phosphate, hence the name) attached to a 3-carbon glycerol backbone. The hydroxyl groups of the glycerol are esterified by long fatty acid chains. The phosphate end of the molecule is hydrophilic while the fatty acids are hydrophobic. When placed in an aqueous environment, these phospholipids spontaneously form a lipid bilayer with the hydrophilic portion facing the aqueous environment and the hydrophobic portion embedded in the middle of the bilayer. Thus, the cell membrane essentially consists of these lipid bilayers with proteins embedded in them. Some proteins span the membrane while others are found on the outside or the inside. Many of the membrane-spanning proteins are involved in transport or energy generation. Some of these protein molecules are anchored to structures in or near the membrane while most of them are free to move within the plane of the membrane. Thus, the cell membrane is not a rigid structure but has a “fluid mosaic” structure and is stabilized by H-bonds and hydrophobic interactions between neighboring lipid molecules. Hydrogen bonds can also form between membrane proteins and the lipids. Further stability comes from negative charges
on proteins that form ionic interactions with divalent cations such as Mg\(^{2+}\) and Ca\(^{2+}\) and the hydrophilic head of lipids.

![Dioleoyl phosphatidyl ethanolamine](image)

**Fig. A-1.1** A. Dioleoyl phosphatidyl ethanolamine, a phospholipid. B. Structure of cell membrane.

Covering the cell membrane is the cell wall. Most bacterial cell walls fall into two major categories: Gram-positive and gram-negative (Fig.A-1.2). This is based on Gram staining characteristics that reflect major structural differences between the two groups. The Gram staining procedure involves four basic steps:

<table>
<thead>
<tr>
<th>Gram-Positive Cell Envelope</th>
<th>Gram-Negative Cell Envelope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsule</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>Outer membrane</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>Periplasm</td>
<td>Periplasm</td>
</tr>
<tr>
<td>Cell membrane</td>
<td>Cell membrane</td>
</tr>
</tbody>
</table>

**Fig. A-1.2** Structure of gram-positive and gram-negative cell envelope.
1. The bacteria are first stained with the basic dye, crystal violet. Both gram-positive and gram-negative bacteria become directly stained and appear purple after this step.

2. The bacteria are then treated with “Gram’s iodine solution”. This allows the stain to be retained better by forming an insoluble crystal violet-iodine complex. Both gram-positive and gram-negative bacteria remain purple after this step.

3. “Gram’s decolorizer”, a mixture of ethyl alcohol and acetone, is then added. This is the differential step. Gram-positive bacteria retain the crystal violet-iodine complex while gram-negative are decolorized.

4. Finally, the counterstain safranin (also a basic dye) is applied. Since the gram-positive bacteria are already stained purple, they are not affected by the counterstain. Gram-negative bacteria, which are now colorless, become directly stained by the safranin.

Other types of cell walls are found in a few bacterial species (neither gram-positive nor gram-negative). Common gram-positive bacteria of medical importance include *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Clostridium species*. Common Gram-negative bacteria of medical importance include *Salmonella species*, *Shigella species*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*,...
Hemophilus influenzae, Escherichia coli, Klebsiella pneumoniae, Proteus species, and Pseudomonas aeruginosa.

**Gram-Positive Cell Wall**

The cell wall of gram-positive bacteria is primarily made of peptidoglycan with attached accessories like teichoic acids, teichuronic acids, polyphosphates or carbohydrates. The peptidoglycan is a single, highly cross-linked macromolecule that surrounds the bacterial cell membrane and provides rigidity (Fig. A-1.4). It consists of a glycan (polysaccharide) backbone consisting of the repeating disaccharide N-acetylmuramic acid (β1→4) N-acetylglucosamine. The glycan strands vary in length and are estimated to contain 5 – 30 subunits depending on the bacterial species. The C-3 of N-acetylmuramic acid (MurNAc) is substituted with a lactyl ether group derived from pyruvate. The lactyl ether connects the glycan backbone to a peptide side chain that contains L-alanine, (L-Ala), D-glutamate (D-Glu), diaminopimelic acid (DAP), and D-alanine (D-Ala). The side chains may be cross-linked by peptide bridges that vary in structure among bacterial species.

The peptidoglycan molecules are covalently attached to anionic polymers through the “linkage unit”, which consists of the disaccharide ManNAc(β1→4)GlcNAc (Fig. A-1.4). Although many gram-positive bacteria are known to synthesize identical linkage units, some species modify its structure with other carbohydrates whereas a few others use entirely different compounds to tether their wall teichoic acids. The teichoic acid, which extends through and beyond the cell wall, consists of polyglycerol phosphate, poly-ribitol phosphate or poly-glucosyl phosphate all of which may be glycosylated and/or amino acid esterified. Some have lipids
attached to it and are known as lipoteichoic acid. Teichuronic acids are acidic polysaccharides containing uronic acids (e.g., glucuronic acid) and other sugars.

**A.**

![Structure of peptidoglycan](image)

**B.**

![Teichoic acid linkage unit](image)

**Fig. A-1.4 A) Structure of peptidoglycan. B) Peptidoglycan connected to teichoic acid through the linkage unit.**
Gram-Negative Cell Wall

The gram-negative cell wall consists of a thin layer of peptidoglycan and an outer membrane made of lipopolysaccharide (LPS) as well as lipids and proteins (Fig. A-1.3). In between the cell membrane and peptidoglycan, there is a large periplasmic space. The periplasmic space is about 20 to 40% of total volume of cells grown under typical conditions. It contains several detoxifying (e.g. β-lactamase) and degradative (e.g. phosphatase, protease, endonuclease) enzymes, cytochromes, chemical sensors and binding proteins involved in transport of amino acids, sugars, phosphate, sulfate etc.

![Diagram of Lipopolysaccharide](image)

**Fig. A-1.5 Lipopolysaccharide**

The outer membrane of the cell wall can be further divided into outer and inner leaflet. The inner leaflet of the outer membrane is similar to cytoplasmic membrane and is made up of phospholipids and lipoproteins that connect the outer membrane to the peptidoglycan. The outer leaflet of the outer membrane is made up of lipopolysaccharides (LPS). An LPS molecule consists of a lipid portion called “lipid A” embedded in the membrane and a polysaccharide portion extending outward from the bacterial surface (Fig. A-1.5). Lipid A component of LPS (also called endotoxin) is responsible for endotoxicity effects of LPS on the host. The structure
of lipid A is highly conserved among gram-negative bacteria and consists of phosphorylated N-acetylglucosamine (GlcNAc) dimer with 6 or 7 saturated fatty acid chains attached to it. Some fatty acids are directly attached to the GlcNAc dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The polysaccharide portion of LPS consists of a short, branched “core (R) antigen” or “R-polysaccharide” that is common to all gram-negative bacteria and a long chain called the “O-antigen”. The “core” structure is attached to the C-6 of one of the GlcNAc in lipid A and consists of heptose, ketodeoxyoctanoic acid (KDO), glucose and glucosamine sugars. The somatic O-antigen or O-polysaccharide is attached to the core polysaccharide and consists of repeating oligosaccharide subunits of 3 – 5 sugars. The O-antigen is usually much longer than the R-antigen and may contain up to 40 repeating units. The loss of O-antigen results in the loss of virulence of the bacteria suggesting that this portion is important for host-parasite interaction while the loss of parts of the core portion makes the bacteria sensitive to a range of hydrophobic compounds, including antibiotics, detergents, bile salts and mutagens. The core region of LPS contains a large number of charged groups and is thought to be important in maintaining the permeability properties of the outer membrane.

In bacteria belonging to the enterobacteriacea family, there is a polysaccharide, enterobacterial common antigen (ECA), covalently attached to the phospholipids of the inner leaflet of the outer membrane. In a few bacteria, the polysaccharide is attached to the lipid A of LPS. The biological significance of ECA has not been clarified, but it is indicated that ECA is needed for the expression of the full pathogenic capacity of the bacteria. The repeating unit of ECA isolated from different sources have been found to have repeating units of the following trisccharide:

\[
\rightarrow 3)-\alpha-D-Fuc4NAc-(1\rightarrow 4)-\beta-D-ManNAcUA-(1\rightarrow 4)-\alpha-D-GlcNAc-(1\rightarrow
\]
Capsule (K-antigen)

A fundamental requirement for most pathogenic bacteria that enter the human body is to escape phagocytosis by macrophages or polymorphonuclear phagocytes. The most common means utilized by bacteria to avoid phagocytosis is an antiphagocytic capsule. The capsule is a major virulence factor, e.g. all of the principal pathogens which cause pneumonia and meningitis, including *Haemophilus influenzae*, *Neisseria meningitidis*, *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and group B streptococci have polysaccharide capsules on their surface. Nonencapsulated mutants of these organisms are avirulent.

The chemical nature of the capsule is important in the functions the capsule plays in the infection process. The capsules of bacteria are chemically diverse but the majority of them are polysaccharide in nature. These polymers are composed of repeating oligosaccharide units of two to four monosaccharides. Some may contain acetic acid, pyruvic acid and/or the methyl esters of hexoses. At least two species of pathogenic bacteria produce protein capsules; *Bacillus anthracis* produces a capsule of pure D-glutamic acid and *Yersinia pestis* produces a capsule of mixed amino acids. Capsules may be weakly antigenic to strongly antigenic, depending on their chemical complexity. They may be covalently linked to the underlying cell wall or just loosely bound to it. Not all bacteria form capsules but in those that do the capsule is the interface between the bacterial cell and the external environment.
Appendix –2

Overexpression of Proteins using Plasmids Constructed from pET Vectors

A vector is a DNA molecule known to replicate autonomously in a host cell, to which a segment of DNA (a gene) may be inserted to allow its replication. The product obtained by inserting a gene or a segment of DNA into a vector is called a plasmid. Plasmids constructed from vectors of the pET series are known for their high levels of over-expression of protein. Furthermore, the over-expression is regulated. A general map of a pET 11 vector is shown in Fig. A-2.1.

Fig. A-2.1 A general map of a pET vector. “Amp” represents the gene that codes for ampicillin resistance. “ColE1ori” is the origin of replication. MCS = multiple cloning site

Fig. A-2.2 The steps involved in converting the information in DNA into protein.
The regulation of gene expression is achieved by cloning the gene of interest downstream of the T7 promoter (at the multiple cloning site shown in figure 1). A promoter is a DNA sequence to which RNA polymerase may bind and initiate transcription (converting the information in DNA into RNA, (Fig. A-2.2)). The transcription of a gene inserted downstream of the T7 promoter is initiated only when T7 RNA polymerase from the host cell binds to the T7 promoter. The DE3 strains of \textit{E. coli} posses a chromosomal copy of the T7 RNA polymerase gene under the control of the \textit{lacUV5} promoter. Thus the \textit{lacUV5} promoter controls the expression of the polymerase and hence the gene of interest. In the absence of lactose in the growth media, the expression of the T7 RNA polymerase is repressed. However when an inducer molecule like allolactose or IPTG (isopropylthio-\(\beta\)-galactoside) (Fig. A-2.3) is added to the media, it binds to the \textit{lacUV5} promoter and induces the expression of T7 RNA polymerase. The polymerase in turn binds to the T7 promoter on the plasmid and induces the high-level transcription of the gene of interest.\(^{127}\)

![Structure of inducer molecules](Image)

\textbf{Fig. A-2.3 Structure of inducer molecules}
Appendix-3

**L-Ru5P 4-epimerase and araD sequence**

```
MLEDLKRQVLEANLALPKHN
ATGTATAAGGATCTCAAAAGGCAGGTATTTAGGAAACCTGCTGCAGGATACAAAC
TACAAATCTACAGTTTCGGTGCATATATCTTCGGTTGAGCGTTGACGGACGGGCTTGGTGG
LVTLTWGNVSAVDREGERGVFV
CTGTACGCTCATGGTGACGGACGGCTGTTGATCGCGGCTGGCTCTGCTGGAAAGCC
IKPSGVDSVMTADDMDVVS
ATCAAACTTCGGTGTCATGACGCTGAGTGAAAAGCCCTCCTGCCACTACGCACTAC
IETGEVVADVKEGTKKPSSDTPT
ATCGAAACCCTTAAAGCTTCGGTGCACTGACATGATGGTACGGACGGCCTACA
RLYQAFPSIGGIVHTHSRRH
CGGTCGCTATTAGCCGGCTCCTGCCATTGGCCGATTGTCATAGCCGCTGCCAC
ATIWAQAGQSIPTAGTTTHAD
GCCACCATTGGCGCGCGCTGATTTCCAAGACCACCACGCAGAAG
YFGTIPCTKRMTDAEINGE
TATTTACGGCCAAATGGTACGGCAGCAAATGCCGCAAAATGCCG
ATAAATGCGCTGGTAAGGGTGGCGTTTTACTGGTTGGTGCGCTT
```
Y E W E T G N V I V E T F E K Q G I D A 160
TAT GAG TGG GAA ACC GGT AAC GTC ATC GTA GAA ACC TTT GAA AAA CAG GGT ATC GAT GCA 480
ATA CTC ACC CTT TGG CCA TTG CAG TAG CAT CTT TGG AAA CTT TTT GTC CCA TAG CTA CGT

A Q M P G V L V H S H G P F A W G K N A 180
GCG CAA ATG CCC GGC GTT CTG GTG CAT TCC CAC GGC CCG TTT GCA TGG GGC AAA AAT GCC 540
CGC GTT TAC GGG CCG CAA GAC CAG GTA AGG GTG CCG GGC AAA CGT ACC CCG TTT TTA CGG

E D A V H N A I V L E E V A Y M G I F C 200
GAA GAT GCG GTG CAT AAC GCC ATC GTG CTG GAA GAT GCG GTT TAT ATG GGG ATA TTA TGC 600
CTT CTA CCG CAC GCA TTG CCG TAG CAC GAC CTT CTC CAG CGA ATA TAC CCC TAT AAG ACG

R Q L A P Q L P D M Q Q T L L D K H Y L 220
CGT CAG TTA GCG CCG CAG TTA CCG GAT ATG CAG CAA ACG CTG CTG GAT AAA CAC TAT CTG 660
GCA GTC AAT CGC GGC GTC AAT GCC GTA TAC GTC GTT GTC GAC GAC CTA TTT GTG ATA GAC

R K H G A K A Y Y G Q END 231
CGT AAG CAT GCC GCG AAG GCA TAT TAC GGG CAG TAA 696
GCA TTC GTA CCG CCG TTC GGT ATA ATG CCC GTC ATT
Appendix-4

UDP-N-Acetylglucosamine 2-Epimerase and nfrC Sequence

MKVLTVFGTRPEAIKMAPLV

ATG AAA GTA CTG ACT GTA TTT GGT ACG CGC CCG GAA GCC ATC AAG ATG GCG CCG TTG GTG

GAC TTT CAT GAC TGA CAT AAA CCA TGC GCG GGC CTT CGG TAG TTC TAC GCC GGC AAC CAC

HALAKDPFFEAAKVCVTAQHR

CAT GCG TTG GCA AAA GAT CCT TTT TTT GAG GCT AAA GTT TGC ACT GCG CAG CAT CGG

GTA GCG AAC CGT TTT CTA GGA AAA AAA CTC CGA TTT CAA AGC CAG TGA GCC GTC GTA GCC

EMLDQVLLKLFISIVPDYDLNI

GAG ATG CTC GAT CAG GTG CTG AAA CTC TTT TCC ATT GTA CCT GAC TAC GAT CTC AAC ATA

CTC TAC GAG CTA GTC CAC GAC TTT GAG AAA AGG TAA CAT GGA CTG ATG CTA GAG TTA TAT

MQPGQLTEITCRILEGLK

ATG CAG CCA GGA CAG GCC CTG ACA GAG ATA ACC TGT CGG ATT CTG GAA GGG CTA AAA CCT

TAC GTC GGT CCT GTC CCG GAC TGT CTC TAT TGG ACA GCC TAA GAC CTT CCC GAT TTT GGA

ILAEFKPDVVLVHGDT-TTL

ATT CTT GCC GAG TTC AAA CCA GAC GTC GTG CTG GTT CAC GGC GAT ACG ACG ACG ACTG

TAA GAA CGG CTC AAG TTT GGT CTG CAG CAC GAC CAA GTG CCG CTA TGC TGC TGC GAC

ATSŁALAFYQRIPVGHVE

GCA ACC AGC CTG GCG GCG TTT TAT CAG CGT ATT CCT GTT GTG CAC GGT GAT ACC GCG AGC

AAG TTA TAA GTA GCA TAA GGA CAA CCA GTG CAA CTC CGA CCA GAC

RTGDLYSWPEEANRTL

CGC ACG GCC GAT CTC TAT TCG CCG TGG CCG GAA GAG GCT AAC CGT ACA TTG ACC GGG CAT
GCG TGC CCG CTA GAG ATA AGC GGC ACC GGC CTT CTC CGA TTG GCA TGT AAC TGG CCC GTA

L A M Y H F S P T E T S R Q N L L R E N 160
CTG GCG ATG TAT CAC TTC TCT CCA ACC GAA ACT TCC CGG CAA AAC TTG CTG CGT GAA AAC 480
GAC CGC TAC ATA GTG AAG AGA GGT TGG CTT TGA AGG GCC GTT TTG AAC GAC GCA CTT TTG

V A D S R I F I T G N T V I D A L L W V 180
GTT GCG GAT AAG CGA ATC TTC ATT ACC GGT AAT ACA GTC ATT GAT GCA CTG TTA TGG GTG 540
CAA CGC CTA TCG GCT TAG AAG TAA TGG CCA TTA TGT CAG TAA CTA CGT GAC AAT ACC CAC

R D Q V M S S D K L R S E L A A M Y P F 200
CGT GAC CAG GTG ATG ACG ACC AAC AAG CTG CGT TGA GAA CTG GCG GCA AAT TAC CCG TTG 600
GCA CTG GTC CAC TAC TCG TCG CTC GCC GCA AGT CTT GAC CGC CGT TTA ATG GGC AAA

G F E E I C H A L A D I A T T H Q D I Q 240
GCC TTT GAA GCA ATC TGC CAC CGG CTG GCA GAC ATC GCC ACC ACC CAG CAC GAC ATC CAG 720
CCG AAA CTT CTT TAG ACG GTG GCC GAC CTG CTG TAG CGG TGG TGC GTC CTG TAG GTC

I V Y P V H L N P N V R E P V N R I L G 260
ATT GTC TAT CCG GTG CAT CTC AAC CCG AAG GTC AGA GAA CCG GTC AAT CGC ATT CTG GGG 780
TAA CAG ATA GGC CAC GTA GAG TTG GGC TTG CAG TCT CTT GCC CAG TTA GCG TAA GAC CCC

H V H N V I L I D P Q E Y L P F V W L M 280
CAT GTG AAG GAT GTC ATT CTG ATC GAT CCC CAG GAG TAT TTA CCG TTT GTC TGG CTG ATG 840
GTA CAC TTT TTA CAG TAA GAC TAG CTA GGG GTC CTC ATA AAT GGC AAA CAG ACC GAC TAC
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