MECHANISTIC STUDIES ON
ADP-1-GLYCERO-d-MANNO-HEPTOSE 6-EPIMERASE AND
UDP-N-ACETYLGLUCOSAMINE 5-INVERTING 4,6-DEHYDRATASE

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ABSTRACT

ADP-L-glycero-D-manno-heptose 6-epimerase (HldD) catalyzes the inversion of configuration at C-6′′ of the heptose moiety of ADP-D-glycero-D-manno-heptose and ADP-L-glycero-D-manno-heptose. HldD operates in the biosynthesis of L-glycero-D-manno-heptose, a conserved sugar in the core region of lipopolysaccharide (LPS) of Gram-negative bacteria. This work supports a direct redox mechanism whereby HldD uses its tightly bound NADP⁺ to oxidize the substrate at C-6′′, generating a ketone intermediate. Reduction from the opposite face generates the epimeric product. An analog of the ketone intermediate, ADP-β-D-manno-hexodialdose 8, was shown to undergo dismutation giving equal amounts of ADP-mannose 9 and ADP-mannuronate 10. Observation of transient NADPH during dismutation established participation of the tightly bound cofactor.

Further studies address how HldD is able to access both faces of the ketone intermediate with correct alignment of NADPH, the ketone intermediate, and a catalytic acid/base residue. It is proposed that Escherichia coli K-12 HldD contains two catalytic acid/base residues, tyrosine 140 and lysine 178, each of which facilitates redox chemistry on opposite faces of the ketone intermediate. The ketone intermediate may access either base via rotation about the C-5′′/C-6′′ bond. The observation that two single mutants, Y140F and K178M, have severely compromised epimerase activities, yet retain dismutase activity, supports this hypothesis.

UDP-N-acetylglucosamine 5-inverting 4,6-dehydratase (PseB) is a unique sugar nucleotide dehydratase that inverts the C-5′′ stereocentre during conversion of UDP-N-acetylglucosamine to UDP-2-acetyl-2,6-dideoxy-β-L-arabino-4-hexulose. PseB catalyses the first step in the biosynthesis of pseudaminic acid, which is found as a post-translational modification on the flagellin of Campylobacter jejuni and Helicobacter pylori. PseB uses its tightly bound NADP⁺ to oxidize UDP-GlcNAc at C-4′′, enabling dehydration. The α,β
unsaturated ketone intermediate thus generated is reduced by delivery of a hydride from NADPH to C-6", and a proton to C-5". Consistent with this mechanism, a solvent derived deuterium becomes incorporated into the C-5" position of product during catalysis in D₂O. Likewise, PseB catalyzes solvent isotope exchange into the H5" position of the product, and the elimination of HF from UDP-6-deoxy-6-fluoro-GlcNAc 23. Mutants of the putative catalytic residues aspartate 126, lysine 127 and tyrosine 135 have severely compromised dehydratase, solvent isotope exchange, and HF elimination activities.
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Figure 3.71 Synthesis of mannose dialdehyde 20. a) TMSCl, pyridine. b) CrO₃, pyridine, CH₂Cl₂. c) K₂CO₃, MeOH.

Figure 3.72 Partial ¹H NMR spectrum (the ‘anomeric region’) of 20 suggests that 20 exists as a complex mixture in aqueous solution (400 MHz, D₂O).

Figure 3.73 Possible forms of 20 in aqueous solution.

Figure 3.74 Dismutation of mannose dialdehyde 20 monitored by ¹H NMR spectroscopy (400 MHz). Top spectrum: Control containing 18.4 mM AMP and 8.5 mM 20 in 100 mM potassium phosphate pH 8.0 deuterated buffer after 21 h incubation at 37 °C. Middle spectrum: Test reaction with 75 µM HldD after 21 h incubation at 37 °C. Bottom spectrum: Test reaction after incubation with 1.8 µmol mannose added (enhancing mannose concentration by 3.7 mM).

Figure 3.75 Mannose dialdehyde 20 and AMP may bind simultaneously to HldD, mimicking ADP-β-D-manno-hexodialdose 8 in the active site.
Figure 3.76  A) The natural substrate for the kinase activity of HldE is D,D-Hep 7P. B) It is proposed that HldE may accept mannose for the kinase activity when phosphate buffer is present. This would generate mannose 1-phosphate which is known to be accepted by the adenylyltransferase activity of HldE. ADP-mannose would be made from D-mannose by HldE without phosphatase GmhB.

Figure 3.77 Relative positions of candidate catalytic acid/base residues (black), NADP$^+$ (grey) and ADP-α-D-glucose (grey) derived from the 2.0 Å X-ray crystal structure of HldD (Protein Data Bank accession number 1EQ2). Image created with Swiss-PdbViewer.

Figure 3.78 Partial $^1$H NMR spectra during the epimerization of 2.7 mM ADP-D,D-Hep to ADP-L,D-Hep (400 MHz, 10 mM potassium phosphate, pH 7, 23 °C, D$_2$O). A) Epimerization catalyzed by 2.65 µM wild-type HldD. B) Epimerization catalyzed by 2.65 µM Y140F HldD.

Figure 3.79 HPLC chromatograms of time points taken during the dismutation of ADP-manno-hexodialdose 8 (injected: 50 µL of 1 mM ADP-sugar solution). A) ADP-manno-hexodialdose control incubated for 2 h at 37 °C. B) Product mixture after 60 min (37 °C) incubation with WT HldD. C) Product mixture after 2 h (37 °C) incubation with Y140F HldD.

Figure 3.80 Two-base mechanism of HldD with tyrosine 140 and lysine 178 playing the roles of catalytic acid/base residues. The stereospecific roles shown here for illustrative purposes are based upon examination of the crystal structure. Further studies are required to make this distinction.

Figure 3.81 Strategy to identify stereospecific roles of each catalytic base.

Figure 3.82 Catalytic strategy to identify stereospecific roles of catalytic bases using ADP-manno-hexodialdose to reduce tightly bound NADP$^+$ in situ.

Figure 4.83 Proposed mechanism of PseB.

Figure 4.84 PseB is proposed to catalyze isotopic exchange at H5'' of UDP-arabino-sugar.

Figure 4.85 PseB is proposed to eliminate HF from UDP-6-deoxy-6-fluoro-GlcNAc.

Figure 4.86 SDS-PAGE of recombinant His-tagged PseB purified by affinity chromatography. Lanes: 1 and 2) molecular weight standards BSA (29 kDa) and carbonic anhydrase (66 kDa). 2) His-tagged wild-type PseB. 3) D126N. 4) K127A. 5) Y135F.

Figure 4.87 Partial UV spectrum of 128 µM D126N in 20 mM pH 9.0 Tris before and after the addition of 500 nmol sodium borohydride (to give 1.2 mM sodium borohydride).

Figure 4.88 A) In situ oxidation of tightly bound NADPH with UDP-4-keto-6-deoxy-GlcNAc. B) UDP-4-keto-6-deoxy-GlcNAc is the product of PgIF-catalyzed dehydration of UDP-GlcNAc.
Figure 4.89 Partial $^1$H NMR spectra during dehydration of 12.1 mM UDP-GlcNAc to UDP-arabino-sugar as catalyzed by 18 µM PseB at (400 MHz, $D_2O$, RT). A) UDP-GlcNAc in 10 mM potassium phosphate pH 7.0. B) Five minutes after the addition of PseB (9.7 % conversion). C) 90 min after the addition of PseB (74 % conversion). The small signal marked with an asterix (*) indicates the appearance of a new product after 90 min. D) Partial $^1$H NMR spectrum of UDP-arabino-sugar prepared in $H_2O$ showing strong H$^5$/H$^6$" coupling (J$^5$,6" = 7.6 Hz).

Figure 4.90 PseB catalyzed dehydration of UDP-GlcNAc in D$_2$O produces UDP-[5$^2$H]-arabino-sugar, which exists in the unhydrated and hydrated forms in aqueous solution. In addition, PseB catalyzes epimerization of the UDP-arabino-sugar to UDP-4-keto-6-deoxy-GlcNAc, which also exists in the unhydrated and hydrated forms in aqueous solution.

Figure 4.91 Partial $^1$H NMR spectra demonstrating the PseB-catalyzed nature of the epimerization of UDP-arabino-sugar ($\odot$ = unhydrated, $\bullet$ = hydrated) to UDP-4-keto-6-deoxy-GlcNAc ($\Delta$ = unhydrated, $\blacksquare$ = hydrated)(total UDP-sugar concentration = 4.1 mM, pH 7.0 potassium phosphate, $D_2O$, RT), in which spectra A-D) document the incubation with 91 µM PseB and spectrum, E) is the control with no enzyme. Spectra A-C) acquired at 300 MHz, spectra D, E) acquired at 400 MHz.

Figure 4.92 Partial $^1$H NMR spectra monitoring solvent isotopic exchange into the UDP-arabino-sugar catalyzed by 11.5 µM His-tagged wild-type PseB ($\odot$ and $\oplus$ are downfield H$^6$" signals of non deuterated sugars, $\bullet$ and $\blacksquare$ are H$^6$" signals of non-deuterated and overlapping [2H5'] sugars)(Total concentration of UDP-sugars = 10.5 mM, 56 % UDP-arabino-sugar and 44 % UDP-4-keto-6-deoxy-GlcNAc at t = 0, 10 mM pH 7.0 potassium phosphate, $D_2O$, 400 MHz).

Figure 4.93 Synthesis of UDP 6-deoxy-6-fluoro-GlcNAc 23. a) TrCl, pyr., then Ac$_2$O. b) 50 psi H$_2$, Pd(OH)$_2$/C. c) DAST, CH$_2$Cl$_2$. d) Me$_2$NH. e) Et$_2$NP(OBn)$_2$, triazole. f) H$_2$O$_2$. g) H$_2$, Pd/C. h) NaOCH$_3$. i) UMP morpholidate, tetrazole.

Figure 4.94 $^{19}$F NMR spectra during the incubation of 5 mM UDP-6-deoxy-6-fluoro-GlcNAc 23 with 25 µM PseB in 50 mM potassium phosphate pH 7.0 (282.4 MHz, $D_2O$). A) Spectrum immediately after addition of PseB (t = 0). B) After 18 h incubation at 37 °C. C) After addition of 2.4 µmol NaF to incubated sample.

Figure 4.95 Proposed suicide inactivation of PseB by UDP-6-deoxy-6,6-difluoro-GlcNAc 25. Ms. Jackie Bassiri found that incubation of 25 with PseB did not result in any observable changes to 25 or to PseB.

Figure 4.96 Proposed future study of the stereochemistry of the reduction step of PseB.

Figure A.97 $^1$H NMR spectrum of adenosine diphosphate 6-deoxy-6-fluoro-$\beta$-D-mannose 1, bis(triethylamine) salt ($D_2O$, 300 MHz).

Figure A.98 $^1$H NMR spectrum of adenosine $\beta$-D-manno-hexodialdose diphosphate 8, bis(triethylamine) salt ($D_2O$, 400 MHz).
Figure A.99 $^1$H NMR spectrum of adenosine $\beta$-D-mannose diphosphate 9, bis(triethylamine) salt ($D_2O$, 400 MHz).

Figure A.100 $^1$H NMR spectrum of adenosine $\beta$-D-mannuronate diphosphate 10, bis(triethylamine) salt ($D_2O$, 400 MHz).

Figure A.101 $^1$H NMR spectrum of mannose dialdehyde 20 ($D_2O$, 400 MHz).

Figure A.102 $^1$H NMR spectrum of adenosine 6-azido-6-deoxy-$\beta$-D-mannose diphosphate 22, bis(triethylamine) salt ($D_2O$, 400 MHz).
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<td>$\delta$</td>
<td>chemical shift (ppm)</td>
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<tr>
<td>A</td>
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<tr>
<td>$A_{260}$</td>
<td>absorbance at 260 nm</td>
</tr>
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<td>Ac</td>
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<td>adenosine 5'-triphosphate</td>
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<td>cytidine</td>
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<td>$N$-cyclohexyl-$N'$(2-morpholinoethyl)carbodiimide methyl-$p$-toluenesulfonate</td>
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<td>camphorsulfonic acid</td>
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<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
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$J$ coupling constant (Hz)
Kdo 3-deoxy-D-manno-oct-2-ulosonic acid
lac operon operon required for the transport and metabolism of lactose
LB Luria-Bertani
LIC ligation-independent cloning
LPS lipopolysaccharide
m multiplet
MALDI-TOF matrix-assisted laser desorption/ionization time-of-flight
ManNAc $N$-acetylmannosamine
MW molecular weight
NDP nucleotide diphosphate (any nucleotide)
NAD$^+$, NADH nicotinamine adenine dinucleotide, and its reduced form
NADP$^+$, NADPH nicotinamine adenine dinucleotide phosphate, and its reduced form
NAPS nucleic acid protein service
NMR nuclear magnetic resonance
NOE nuclear Overhauser effect
NovW dTDP-6-deoxy-D-xylo-4-hexulose 3-epimerase
OD$_{600}$ optical density at 600 nm
PAGE polyacrylamide gel electrophoresis
PCR polymerase chain reaction
PEP phosphoenolpyruvate
PglF UDP-$N$-acetyl-glucosamine 4,6-dehydratase (cj1120c, non-inverting)
PIX positional isotope exchange
PLP pyridoxal 5′-phosphate
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<td>UDP-2-acetyl-2,6-dideoxy-(\beta)-L-arabino-4-hexulose aminotransferase</td>
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<td>PseF</td>
<td>CMP-pseudaminic acid synthase</td>
</tr>
<tr>
<td>PseG</td>
<td>UDP-2,4-diacetamido-2,4,6-trideoxy-L-altrose hydrolase</td>
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<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>(t)</td>
<td>time</td>
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<tr>
<td>T</td>
<td>thymidine</td>
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<td>TS</td>
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<tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>UDP</td>
<td>uridine 5'-diphosphate</td>
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<td>UDP-2-acetyl-2,6-dideoxy-(\beta)-l-\textit{arabino}-4-hexulose</td>
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<tr>
<td>UMP</td>
<td>uridine 5'-monophosphate</td>
</tr>
<tr>
<td>VT NMR</td>
<td>variable temperature nuclear magnetic resonance</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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Abortive complex  A complex of nonfunctional enzyme with substrate. In the case of NAD(P)^+ -dependent enzymes with tightly-bound cofactor that operate by transient oxidation this is the complex of enzyme containing NAD(P)H with substrate bound.

Activated stereocentre  A stereocentre bearing an acidic C-H bond (pK\textsubscript{a} < 30) which an epimerase or racemase could deprotonate directly. Usually activated by the presence of a nearby carbonyl functionality.

Carbohydrates\(^1\)  Originally, compounds such as aldoses and ketoses, having the stoichiometric formula C\(_n\)(H\(_2\)O)\(_n\), hence ‘hydrates of carbon’. The generic term carbohydrate includes monosaccharides, oligosaccharides and polysaccharides as well as substances derived from monosaccharides by reduction of the carbonyl group (alditols), by oxidation of one or more terminal groups to carboxylic acids, or by replacement of one or more hydroxyl group(s) by a hydrogen atom, an amino group, thiol group or similar groups. It also includes derivatives of these compounds.

Nonstereospecific  As enzymes typically catalyze stereospecific reactions (see definition of stereospecific below), the term nonstereospecific has been adopted by enzymologists to describe enzyme catalyzed reactions in which the reaction is not stereospecific (such as epimerizations). This term simply highlights that the reaction is somewhat unusual in enzymology.
Stereospecific

A reaction is termed stereospecific if, in such a reaction, starting materials differing only in their configuration are converted to stereoisomerically distinct products. According to this definition, a stereospecific process is necessarily stereoselective, but stereoselectivity does not necessarily imply stereospecificity.

The term may be extended to a process involving a chiral catalyst (including an enzyme) or chiral reagent when the configuration of the product of the reaction depends uniquely on the configuration of the catalyst or reagent; i.e. becomes reversed when a catalyst or reagent of the opposite configuration is employed.

Tightly bound

In reference to a cofactor bound to an enzyme this means that the cofactor is strongly held through non-covalent binding to the enzyme such that it is non-exchangeable. Cofactors tightly bound by enzymes are often co-purified with the enzyme and the cofactor will not dissociate from the enzyme during dialysis, centrifugal filtration, or during catalysis. To dissociate the cofactor the enzyme must be denatured.

Unactivated stereocentre

A stereocentre lacking an acidic C-H bond (pKₐ < 30) which an epimerase or racemase could deprotonate directly. Due to the absence of an acidic proton, an epimerase or racemase cannot directly deprotonate an unactivated stereocentre, and must use an alternate mechanism.
ACKNOWLEDGEMENT

Thank you everyone who has helped during the research and writing of this thesis. Foremost, I would like to thank Martin Tanner for accepting me into his lab, supporting me, and creating opportunity. Martin is an excellent mentor; he took time to lend his instruction and wisdom when it was needed, but also allowed me freedom to pursue my own ideas. I am fortunate to have worked with a supervisor who is able to balance these two qualities, and all the while maintain a wonderfully witty sense of humour. The ‘Life Lessons’ will not be forgotten. I would also like to thank those who helped instruct me during my first years in the lab, from whom I have learned a great deal, and who made the lab endlessly entertaining and a centre of high drama: Dr. Andrew Murkin, Dr. Wayne Chou, and Dr. Jay Read. I am lucky to have gotten to know several more generations of lab members who have helped me along the way: Feng Liu, Louis Luk, Pavel Glaze, Timin Hadi, Alain Mayer, Stephen Lau, Jackie Bassiri, Xu Li, Jen Johnson, and Raef Ahmed. I would also like to thank Richard Ting for his help and instruction with things that no one else seemed to know about. Thank you also to previous lab members who I may not have met, but whose theses have proven to be invaluable. I am grateful to Dr. Elena Polishchuk, Candice Martins, and Jie Chen for accommodating me at Biological Services and lending a helping hand when I needed it, and to Dr. Nick Burlinson of the NMR Facility for his help. I am also grateful to the Withers group for use of the fluoride electrode and UV/Vis spectrophotometer, and to the Perrin group for use of the UV/Vis spectrophotometer.
Chapter One
Sugar Nucleotide Epimerases and Dehydratases
1.1 Introduction

This thesis describes the mechanistic study of two enzymes. The chemistry catalyzed by each enzyme is distinct from the other, but these enzymes share a mechanistic strategy of transiently oxidizing their substrates during catalysis. They also operate on similar substrates, and are involved in the biosynthesis of unusual sugars.

Many of the sugars discussed in this thesis are unusual, and perhaps unfamiliar. This is not unexpected, as carbohydrates are a class of molecules that contain an enormous number of individual structures. Nature utilizes many of these carbohydrate structures for a variety of functions that includes the storage of metabolic energy, structural roles, and roles in cellular signalling. While many of these structures are made from common monomers, such as glucose, and differ only in their connectivity and chain length, other natural structures are made from more exotic sugars. These exotic sugars are often involved in recognition events, and their importance is only beginning to be appreciated.

Each exotic sugar has its own biosynthetic pathway, and the strategies that Nature uses in the biosynthesis of these exotic sugars are often fascinating. From an energetic point of view, the biosyntheses of unusual sugars are often quite streamlined. Rather than synthesize each unusual sugar from simple starting materials (de novo biosynthesis), for example as in gluconeogenesis (which starts with pyruvate and generates glucose), Nature has developed more efficient routes that start from existing common sugars, and modifies them directly. Many of these modifications occur on a modified form of the sugar known as a sugar nucleotide. The chemical strategies Nature uses to directly modify sugar nucleotides are often completely different than those that a chemist might devise to achieve the same transformation, and the elucidation of these strategies and mechanisms merits investigation.
Two important classes of sugar-modifying enzymes are epimerases and dehydratases. Epimerases invert the stereochemical configuration at a single stereocentre in molecules bearing more than one stereocentre. They differ from racemases in that racemases operate on molecules with only one stereocentre. Dehydratases catalyze a net elimination of water from their substrates. Together, epimerases and dehydratases provide access to a diverse set of epimeric and deoxy sugars.

In this opening chapter the best understood sugar nucleotide epimerases and dehydratases are discussed, with emphasis on chemical mechanism and roles of catalytic residues. Many of these enzymes belong to the short chain dehydrogenase/reductase (SDR) family, and as such a short discussion of this family of enzymes is included. Two enzymes investigated in this thesis are introduced at the end of this chapter, ADP-L-glycero-D-manno-heptose 6-epimerase (HldD) and UDP-N-acetylglucosamine 5-inverting 4,6-dehydratase (PseB). This chapter is anticipated to establish an appreciation that HldD and PseB are unique in this class of enzymes and must operate by unique mechanisms, demanding mechanistic investigation.

### 1.2 Sugar Nucleotides

A sugar nucleotide is a nucleotide linked via a phosphate to the anomeric position of a sugar (Figure 1.1). The nucleotide consists of a diphosphate (sometimes a monophosphate) linked to the $5'$-oxygen of either ribose or $2'$-deoxy-ribose, which in turn is $\alpha$-linked to a base. The base is a purine (adenine or guanine) or a pyrimidine (cytosine, uracil or thymine).
The nucleotide portion of a sugar nucleotide can serve several functions. The negatively charged phosphates obstruct membrane permeability of the molecule, keeping the exotic sugar within the cell. The nucleotide can also serve as an excellent leaving group in subsequent transferase reactions, in which the sugar is transferred to a glycosyl acceptor molecule. Many of the sugar modifying enzymes also bind the nucleotide portion of the sugar, and the identity of the nucleotide serves to differentiate between pools of sugars.

1.3 Epimerization at Activated and Unactivated Stereocentres

Epimerases can be divided into two general types depending on the nature of the substrate. The first type operates at stereocentres bearing relatively acidic C-H bonds, which are termed ‘activated’ stereocentres. The pKₐ of the proton at these activated stereocentres is relatively low (generally < 30) due to an adjacent functionality, such as a carbonyl, which stabilizes the resulting carbanion (Figure 1.2 A). Typically, epimerases that operate at activated stereocentres employ a direct deprotonation/reprotonation mechanism. The second type of epimerase operates at an unactivated stereocentre bearing a C-H bond that is not acidic (pKₐ > 30). Enzymes are not able to directly deprotonate such a non-acidic C-H bond, and so a different mechanistic strategy must be used.
1.4 Short-chain Dehydrogenase/Reductase (SDR) Enzyme Family

Short-chain dehydrogenase/reductase (SDR) enzymes are a well-established family that contain about 3000 members (identified by primary sequence) including species variants, 63 of which have been identified in the human genome. SDR enzymes perform a variety of reactions with the commonality of binding NAD(P)$^+$, performing redox chemistry, and functioning independently of metal ions. The substrate scope of SDR enzymes is quite wide, ranging from lipophilic steroids to charged sugar nucleotides. Despite functional similarities to medium-chain dehydrogenases/reductases and aldo-keto reductases, the SDR enzyme family is distinct.

Most SDR enzymes have a core structure of 250-350 residues in length. Analysis of the primary structure of SDR enzymes has led researchers to describe this enzyme family as being evolutionarily old, due to low levels of sequence identity (15-30 %)(implying a great length of time has allowed sequence divergence to occur). Typically three catalytic residues are conserved: a tyrosine, a lysine and a serine (which can sometimes be replaced by a threonine). Of these, only the tyrosine is strictly conserved, while the lysine is almost always conserved. The tyrosine (around position 150) is typically four residues upstream from the lysine (Tyr-X-
X-X-Lys, where X is any residue), and the serine (or threonine) is typically about 12 residues upstream from the tyrosine. Also, a Gly-X-X-Gly-X-Gly segment characteristic of a Rossman fold (a NAD(P)\(^+\) binding motif) is generally conserved near the N-terminus of the protein.

Despite the low sequence identity of SDR enzymes, all the available crystallographic data indicates that the tertiary structures are highly related.\(^5\) All structures display a related \(\alpha/\beta\) folding pattern with a Rossman fold near the N-terminus of the peptide. Also, there are three dimensional characteristics of the active site and substrate binding region which are conserved, including the relative positions of the catalytic triad.

The typical positions and roles of the catalytic triad side-chains are described in Figure 1.3.\(^4,8\) The general acid/base residue is tyrosine, which is found in proximity to the nicotinamide ring of the cofactor. The pK\(_a\) of the O\(^n\) of the tyrosine is about 7, lowered by the positively charged lysine and the emerging/existing positive charge of the nicotinamide ring. The lysine does not hydrogen bond with the tyrosine directly, but is hydrogen bonded to the hydroxyls of the ribose moiety of the cofactor. The serine is found in proximity to the carbonyl/hydroxyl of the substrate and the O\(^n\) of the tyrosine. The exact role of the serine is unclear, but it is generally thought to position the substrate during catalysis. In addition, the serine may fine tune the pK\(_a\) of the tyrosine and substrate during catalysis. While this briefly summarizes the common positions and accepted roles of the catalytic triad it should be noted, however, that the active sites and mechanisms need not be identical for all SDR enzymes.
1.5 SDR Sugar Nucleotide Epimerases

1.5.1 UDP-galactose 4-Epimerase (GalE)

Perhaps the most studied sugar nucleotide modifying enzyme of the SDR family is UDP-galactose 4-epimerase (GalE), which interconverts UDP-galactose and UDP-glucose (Figure 1.4). GalE is one of three enzymes in the Leloir pathway, the only route through which glucose and galactose are interconverted biologically.\(^9\) GalE was first reported by Leloir in 1951,\(^{10}\) and it has fascinated scientists ever since. Much of the early work dealt with the elucidation of the chemical mechanism, and it was not until the 1970's that the accepted chemical mechanism was established.\(^{11}\) Since then the majority of the studies have addressed the enzyme structure and structural dynamics during catalysis.
GalE was originally termed ‘Waldenase’, inferring a mechanism involving nucleophilic displacement of the C-4" hydroxyl by water.\textsuperscript{12} This mechanism was quickly dismissed with the establishment that the C-4" oxygen and hydrogen do not exchange with solvent during epimerization.\textsuperscript{13-16} It was also established early on that NAD\textsuperscript{+} is ‘tightly bound\textsuperscript{*} by GalE, and that substrate is able to reduce the cofactor to NADH.\textsuperscript{17, 18} While several mechanistic possibilities could account for these observations, it was Maxwell in 1957 who proposed that GalE oxidizes at C-4" to generate a ketone intermediate (Figure 1.5).\textsuperscript{12}

\begin{center}
\begin{align*}
\text{UDP-galactose} & \rightleftharpoons \text{UDP-4-ketoglucose} \rightleftharpoons \text{UDP-glucose} \\
\text{UDP-galactose 4-epimerase (GalE)} \text{ operates via the UDP-4-ketoglucose intermediate.}
\end{align*}
\end{center}

Evidence supporting UDP-4-ketoglucose as the reaction intermediate came later. An important observation was that an \textit{Escherichia coli} GalE-[4-β-\textsuperscript{3}H]NADH complex was able to deliver its tritium label to UDP-4-keto-6-deoxyglucose to produce tritium-labelled UDP-quinovose and UDP-fucose (Figure 1.6).\textsuperscript{19}

\begin{center}
\begin{align*}
\text{UDP-galactose 4-epimerase} & \left[4-\beta-\textsuperscript{3}H\right]\text{NADH} \\
\text{UDP-4-keto-6-deoxyglucose} & \rightleftharpoons \text{UDP-quinovose} \rightleftharpoons \text{UDP-fucose}
\end{align*}
\end{center}

Figure 1.6 Tritium was observed to be transferred from the cofactor to a ketone intermediate analog.

\textsuperscript{*} In reference to a cofactor bound to an enzyme this means that the cofactor is strongly held through non-covalent binding to the enzyme such that it is non-exchangeable. Cofactors tightly bound by enzymes are often co-purified with the enzyme and the cofactor will not dissociate from the enzyme during dialysis, centrifugal filtration, or during catalysis. To dissociate the cofactor the enzyme must be denatured.
Later, it was reported that extended incubation of UDP-glucose with GalE leads to the accumulation of UDP-4-ketoglucose, observable by reduction with NaB\(^3\)H\(_4\) to generate UDP-[4-\(^3\)H]glucose and UDP-[4-\(^3\)H]galactose.\(^{20}\) The accumulation of UDP-4-ketoglucose was correlated to the reversible substrate-induced inactivation of the enzyme.\(^{21, 22}\) The reversible substrate-induced inactivation of the enzyme is due to premature release of the 4-keto intermediate and the accumulation of reduced cofactor (NADH) tightly bound to the epimerase (Figure 1.7). GalE·NADH binds either UDP-galactose or UDP-glucose to form an ‘abortive complex’,\(^\dagger\) in which epimerization cannot occur.

![Diagram of UDP-glucose metabolism](image)

Figure 1.7 Occasional release of UDP-4-ketoglucose from the active site leads to formation of abortive complex. Square brackets indicate species bound to GalE active site.

The final piece of evidence supporting the direct C-4\(^{\prime\prime}\) oxidation mechanism is the observation of a primary kinetic isotope effect.\(^{23, 24}\) Using UDP-[4-\(^3\)H]hexoses, a primary

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\(^\dagger\) A complex of nonfunctional enzyme with substrate. In the case of NAD(P)\(^+\)-dependent enzymes with tightly bound cofactor that operate by transient oxidation this is the complex of enzyme containing NAD(P)H with substrate bound.
kinetic isotope effect in the range of 1.7 to 3.5 for $k_{\text{H}}/k_T$ was found, implicating that the C-4" C-H bond is broken during catalysis.

With the chemical mechanism thus elucidated, the focus of subsequent work turned to understanding how GalE is able to achieve catalysis. In particular, it was puzzling as to how GalE is able to present either face of the carbonyl of the intermediate ketone to the cofactor. This is interesting because it is in contrast to most dehydrogenases that catalyze stereospecific hydride transfers to and from one particular face of a molecule. In contrast, GalE reduces the ketone intermediate without stereochemical preference, and this has been termed ‘nonstereospecific’. Interestingly, while the hydride transfer is nonstereospecific with respect to the substrate, this is not the case with the $\text{NAD}^+$ cofactor. The hydride transfer to and from $\text{NAD}^+$ is stereospecific to the pro-$S$ position on the $B$ side of the nicotinamide ring.\(^{19}\)

In order to understand how GalE is able to nonstereospecifically transfer a hydride to either face of the ketone carbonyl, a key isotopic labelling experiment was performed. It had been proposed that GalE could either: i) release the ketone intermediate into solution such that it rebinds with the other face of the carbonyl exposed to the hydride, or ii) retain the ketone intermediate and reorient it within the active site. To differentiate between these two scenarios UDP-glucose-$d_7$ and UDP-glucose were incubated together with GalE, and the products were assessed using mass spectrometry.\(^{25}\) The first scenario should result in isotopic scrambling, as it is unlikely the intermediate would rebind to the same active site during catalysis. Epimerization would be an intermolecular process, and $d_1$ and $d_6$ species would be observed. The second scenario is an intramolecular process, and only $d_7$ and $d_0$ species would be observed. The actual

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\(^2\) As enzymes typically catalyze stereospecific reactions (see definition of stereospecific in the Glossary), the term ‘nonstereospecific’ has been adopted by enzymologists to describe enzyme catalyzed reactions in which the reaction is not stereospecific (such as epimerizations). This term simply highlights that the reaction is somewhat unusual in enzymology. Aspects of all epimerases and racemases can be described as nonstereospecific.
observation was that isotopic scrambling does not occur during epimerization, indicating that epimerization occurs within a single active site with one NAD\textsuperscript{+} cofactor mediating the reaction.

Insight into how GalE can achieve this nonstereospecific hydride transfer in one active site comes from the study of the binding energies of different parts of substrate and intermediate. It was revealed that the enzyme binds the UDP moiety much more tightly than the sugar moiety.\textsuperscript{26, 27} The standard free energies for the binding of the UDP moiety of substrates to GalE (GalE·NAD\textsuperscript{+}) is about \(-5\) kcal/mol whereas that for the binding of the sugars is about zero. The standard free energies for binding of UDP-4-ketoglucose to the reduced GalE (GalE·NADH) is about \(-7\) kcal/mol whereas that for 4-ketoglucose is about \(-2\) kcal/mol. This indicates that the UDP moiety serves as an anchor for binding of the substrates and the intermediate, and that the sugar moiety has freedom to move within the active site.

Based on the accumulated evidence, a model explaining nonstereospecific GalE catalysis had developed (Figure 1.8).\textsuperscript{26} It was proposed that the sugar moiety of UDP-4-ketoglucose is able to rotate around the bonds linking the UDP moiety to the 4-ketoglucose moiety. A rotation of approximately 180° would allow the cofactor access to either face of the ketone carbonyl, taking advantage of the pseudo C2 symmetry of the 4-ketoglucose moiety.

Figure 1.8 Nonstereospecific intramolecular hydride transfer catalyzed by GalE.
As protein crystallography advanced, GalE was the subject of numerous studies. One major goal was to observe the conformation of substrate bound in the active site and to determine if the sugar moieties of UDP-glucose and UDP-galactose did, in fact, bind as predicted. This necessitated two important crystal structures, each with either UDP-glucose bound or UDP-galactose bound. The structure of \textit{E. coli} GalE•NADH with UDP-glucose bound (abortive complex) was the first of the two important structures to be solved.\textsuperscript{28} This structure looked promising, as both the NADH and UDP-glucose were found to be in proper orientation relative to each other. The crystal structure of GalE with UDP-galactose however, was more elusive. It was not until a double mutant of the epimerase, S124A•Y149F, was used that a crystal structure of GalE•NADH with UDP-galactose bound was attained.\textsuperscript{29} Comparison of the orientation of the sugar moieties in the two crystal structures reveals that the two sugars are indeed rotated dramatically relative to one another as predicted in Figure 1.8. This demonstrates that the active site is indeed large enough for rotation of UDP-4-ketoglucose, and lends credence to the hypothesis that the intermediate does undergo a rotation during epimerization.

The crystal structures of \textit{E. coli} GalE also reveal the positions of the three conserved residues of SDR enzymes: lysine 153, tyrosine 149 and serine 124.\textsuperscript{28, 30, 31} Lysine 153 is hydrogen-bonded to the hydroxyl groups of the nicotinamide riboside portion of the tightly bound NAD\textsuperscript{+}. Solution studies of GalE revealed that lysine 153 plays an important role in enhancing the chemical reactivity of NAD\textsuperscript{+} upon binding of uridine nucleotides in the active site.\textsuperscript{32} The crystal structure of GalE•NADH•UDP-glucose shows tyrosine 149 and serine 124 close to each other, with tyrosine 149 near the nicotinamide of NAD\textsuperscript{+} and serine 124 hydrogen-bonded to the C-4" hydroxyl of the glucose ring.\textsuperscript{28} Tyrosine 149 was found to have a pK\textsubscript{a} of 6.08 in wild-type GalE. This low pK\textsubscript{a} is attributed mainly to the positive electrostatic field created by NAD\textsuperscript{+} and lysine 153 and partly due to hydrogen bonding with serine 124.\textsuperscript{33}
proximity of serine 124 to the C-4'' oxygen of the glucose (2.6 Å) suggests serine 124 is the
catalytic base rather than the more distant tyrosine 149 (4.3 Å away from the C-4''-OH of
glucose). Mutants of either serine 124 or tyrosine 149, however, both display very little
catalytic activity ($k_{cat} = 0.010 \%$ and $0.035 \%$ of wild-type for Y149F and S124A, respectively).  
This led to the conclusion that tyrosine 149 and serine 124 act together as a
diad, with tyrosine providing the driving force for general acid/base catalysis, and serine 124
mediating proton transfer between the C-4'' hydroxyl and the tyrosine (Figure 1.9). While this
appears to be the case in *E. coli* GalE, a crystal structure of human GalE (GalE•NADH•UDP-
glucose) reveals the corresponding conserved residue, tyrosine 157 is in a suitable position to
deprotonate the C-4''-OH of the glucose directly (tyrosine O$^\text{iii}$ within 3.1 Å). It could be the
case that the catalytic residue that interacts directly with the C-4''-OH of substrate is different in
human and *E. coli* GalE. The human GalE active site is different from that of *E. coli* GalE in
other fundamental ways. In particular, human GalE can accommodate UDP-GlcNAc, which *E. coli* GalE cannot. It does appear that in both epimerases the ultimate base is the same
conserved tyrosine, tyrosine 149 in *E. coli* GalE and tyrosine 157 in human GalE.

Figure 1.9 Catalytic diad of *E. coli* UDP-galactose 4-epimerase (GalE).
1.5.2 CDP-tyvelose 2-Epimerase (Tyv)

CDP-tyvelose 2-epimerase (Tyv) catalyzes the interconversion of CDP-tyvelose and CDP-paratose (Figure 1.10). CDP-tyvelose has been identified in some strains of Gram-negative bacteria, and the tyvelose moiety is thought to become incorporated at the nonreducing end of the O-antigen of lipopolysaccharide (LPS), where 3,6-dideoxyhexoses are found.\(^{37}\)

![Figure 1.10 Reaction catalyzed by CDP-tyvelose 2-epimerase (Tyv).](image)

Tyv from *Yersinia pseudotuberculosis* has been the subject of careful mechanistic investigation.\(^{38,39}\) Sequence alignment indicates Tyv belongs to the SDR enzyme family, which prompted investigation of NAD\(^+\) binding to Tyv. It was found that recombinant Tyv tightly binds one NAD\(^+\) per monomer of enzyme, such that it is co-purified with the enzyme. An indication that Tyv uses its cofactor during epimerization is the observation that upon mixing Tyv with CDP-paratose in a stopped flow experiment, a sharp increase in fluorescence corresponding to transiently formed NADH occurs. When the epimerization was carried out in either \(^2\)H\(_2\)O or \(^18\)OH\(_2\) neither \(^2\)H nor \(^18\)O was incorporated into the backbone of CDP-paratose or CDP-tyvelose during epimerization. These observations left two reasonable mechanisms, the first involving transient C-4\(^"\) oxidation with a reversible retro-aldol process (mechanism A, Figure 1.11), and the second involving a direct oxidation/reduction at C-2\(^"\) (mechanism B, Figure 1.12).
Mechanism A (Figure 1.11) was attractive as Tyv shares significant sequence identity (25-30 %) with SDR enzymes known to oxidize at C-4'' of sugar nucleotides. There is also precedent for epimerization at unactivated stereocentres via C-C bond cleavage. Mechanism B (Figure 1.12) is analogous to the mechanism of GalE (Figure 1.8), however the CDP-2-ketoparatose ketone intermediate does not have a pseudo C2 axis of symmetry, and it is not obvious how NADH would be able to readily gain access to either face of the ketone carbonyl.

Initially the authors were misled by an experiment that led them to propose mechanism A is operative. They found that the NADH containing form of the enzyme was able to reduce CDP-4-ketoparatose and generate a mixture of CDP-paratose and CDP-tyvelose. This implied that CDP-4-ketoparatose is an intermediate in the reaction.
The authors sought additional evidence to confirm mechanism A. To this end, CDP-4-deoxy-4-fluoroparatose was synthesized with the anticipation that it would eliminate fluoride and generate a CDP-aldehyde, confirming mechanism A (Figure 1.13). In fact, it was found that CDP-4-deoxy-4-fluoroparatose is a proficient substrate for the epimerase activity of Tyv, strongly supporting mechanism B. With this convincing result, mechanism A quickly went from being favoured to an impossibility. The earlier observation that CDP-4-ketoparatose can be converted into CDP-paratose and CDP-tyvelose by reduced Tyv was explained to be due to an initial aberrant reduction by NADH to generate CDP-paratose and the NAD$^+$ form of Tyv. With active Tyv thus generated, a mixture of CDP-paratose and CDP-tyvelose would then form by the normal epimerization reaction.

Figure 1.13 Anticipated fate of CDP-4-deoxy-4-fluoroparatose assuming mechanism A of Tyv.

To confirm mechanism B, the position of initial oxidation was probed. Using a C-2$''$ and a C-4$''$ deuterated substrate, prolonged incubation led to accumulation of the reduced form of Tyv. This likely occurs by occasional release of the ketone intermediate from the active site and formation of an abortive complex, leaving the cofactor in the NADH form. The tightly bound cofactor was then released and analyzed by mass spectrometry, and it was found that the cofactor isolated from the C-2$''$ deuterated substrate incubation contained 10 % NAD$^2$H, whereas the cofactor isolated from the C-4$''$ deuterated substrate incubation contained 0 % NAD$^3$H. This confirms that oxidation occurs at C-2$''$, supporting mechanism B whereby Tyv generates the CDP-2-keto-paratose intermediate and reduces the carbonyl nonstereospecifically (Figure 1.12).
To gain insight into the putative conformational reorientation that the ketone intermediate must undergo, Tyv from *Salmonella typhi* was investigated with crystallography.\(^{41}\) The crystal structure, containing NAD\(^+\) and CDP, reveals that the conserved lysine, tyrosine and threonine are in positions typical to SDR enzymes, suggesting these residues play similar roles in Tyv. The crystal structure shows that the active site is large enough to accommodate the putative conformational reorientation of the CDP-tyvelose ketone intermediate, however with the lack of a sugar moiety attached to CDP the structure falls short of providing details as to how this occurs.

### 1.5.3 GDP-mannose 3,5-Epimerase (GME)

GDP-mannose 3,5-epimerase (GME) catalyzes the interconversion of GDP-\(\alpha\)-D-mannose with GDP-\(\beta\)-L-galactose and GDP-\(\beta\)-L-gulose (Figure 1.14).\(^{42,43}\) GME is not truly an epimerase, as GDP-\(\alpha\)-D-mannose and GDP-\(\beta\)-L-galactose are diastereomers and not epimers. However, the transformation catalyzed by GME can be viewed as two successive epimerizations, and this process merits discussion here. GME is found in plants and the transformation of GDP-\(\alpha\)-D-mannose to GDP-\(\beta\)-L-galactose is thought to be the first committed step in the *de Novo* biosynthesis of L-ascorbic acid (vitamin C).\(^{42}\) The fate of GDP-\(\beta\)-L-gulose is less certain, however it is thought that it is transformed into L-ascorbic acid through a distinct route.\(^{43}\)

![Figure 1.14 Reactions catalyzed by GDP-mannose 3,5-epimerase (GME).](image-url)
The epimerizations catalyzed by GME are thought to occur via a transient oxidation at C-4" (Figure 1.15).\textsuperscript{44,45} The ketone generated by C-4" oxidation would enhance the acidity of the protons on C-3" and C-5", allowing epimerization via a direct deprotonation/reprotonation mechanism. A final reduction at C-4" regenerates the alcohol. In agreement with this mechanistic proposal it has been observed that GME isolated from the green alga \textit{Chlorella pyrenoidosa} incorporates tritium from \textsuperscript{3}H\textsubscript{2}O into both the C-3" and C-5" positions of substrate and products.\textsuperscript{44}

![Chemical structures and reactions](image)

Figure 1.15 Mechanism of GME.

Recently GME from \textit{Arabidopsis thaliana} has been examined by X-ray crystallography.\textsuperscript{45} Several structures were solved, including several mutants of GME, and with different substrates bound to the active site (GDP-\textalpha-D-mannose, GDP-\textbeta-L-galactose and a mixture of GDP-\textbeta-L-gulose with GDP-13-L-4-ketogulose). The positions of the catalytic triad of lysine, tyrosine and serine are consistent with the typical function and configuration of these residues in SDR enzymes (Figure 1.3). The structures are consistent with tyrosine 174 directly deprotonating the substrate during oxidation at C-4", which is supported by the observation that
a tyrosine mutant (Y174F) was unable to catalyze epimerization. Also in the active site are two residues in appropriate positions to function as the acid/base pair to catalyze epimerization at C-3" and C-5". These residues are cysteine 145 and lysine 217, which are found on opposite sides of the active site with the sugar moiety of the sugar nucleotide sandwiched in between them (Figure 1.16). It appears that cysteine 145 and lysine 217 are able to catalyze both epimerizations, which could be possible by a small lateral movement of the hexose within the active site. The severely hampered activities of several mutants of these residues (C145S, C145A, K127R, K217A) are consistent with this assignment.

![Cysteine 145 and lysine 217 are well positioned to act as the catalytic acid/base pair for C-3" and C-5" epimerizations in GME.](image)

**1.5.4 GDP-4-keto-6-deoxy-mannose 3,5-Epimerase/4-Reductase (GMER)**

GDP-4-keto-6-deoxy-mannose 3,5-epimerase/4-reductase (GMER), also known as GDP-fucose synthase and GDP-fucose synthetase (curiously, given that it does not use ATP) catalyzes the conversion of GDP-4-keto-6-deoxymannose to GDP-L-fucose (Figure 1.17).\textsuperscript{46-48} This is the second step in the \textit{de Novo} biosynthesis of GDP-L-fucose from GDP-mannose. The reaction catalyzed by GMER is not an epimerization, however GMER does catalyze individual epimerization steps during catalysis.
Figure 1.17 Biosynthesis of GDP-L-fucose from GDP-D-mannose.

The mechanism of GMER has not been fully elucidated; however, as the C-3'' and C-5'' positions of GDP-4-keto-6-deoxy-D-mannose are activated stereocentres it would seem likely that GMER catalyzes the stereochemical inversions via a deprotonation/reprotonation mechanism. It has been observed that GMER can catalyze the epimerization steps in absence of NADPH, consistent with a simple deprotonation/reprotonation mechanism.\textsuperscript{49} As is the case with GME, X-ray crystallography revealed two residues in the active site, cysteine 109 and histidine 179 in \textit{E. coli} GMER, which appear to be in suitable positions to catalyze epimerizations at C-3'' and C-5''.\textsuperscript{50} Supporting this assignment, mutants Cys109Ala and His179Asn catalyze the reaction $10^4$ fold slower than wild-type GMER, but still bind NADPH almost as well as wild-type GMER. The reduction step appears to be catalyzed by the conserved triad of lysine 140, tyrosine 136 and serine 107. Each residue is thought to play a typical catalytic function for an SDR enzyme. Consistent with this, it has been demonstrated that GMER transfers a hydride from the \textit{pro-S} position of NADPH during the reduction step, typical of SDR enzymes.\textsuperscript{49}
1.6 Cofactor-independent Sugar Nucleotide Epimerases

1.6.1 Sugar Nucleotide Epimerases Operating at Activated Stereocentres

dTDP-4-dehydrorhamnose 3,5-epimerase (RmIC) inverts the configuration at two stereocentres of dTDP-6-deoxy-D-xylo-4-hexulose to give dTDP-6-deoxy-L-lyxo-4-hexulose (Figure 1.18). While this reaction is technically an isomerisation, it can be viewed as two sequential epimerizations. Two enzymes closely related to RmIC are dTDP-6-deoxy-D-xylo-4-hexulose 3-epimerase (NovW) and dTDP-3-amino-2,3,6-trideoxy-3-C-methyl-D-erythro-hexopyranos-4-ulos 5-epimerase (EvaD). Early work assigned 3,5-epimerase activity to both NovW and EvaD (with dTDP-4-keto-D-glucose), however it has subsequently been demonstrated that NovW only possesses kinetically significant 3-epimerase activity (Figure 1.18), and only the 5-epimerase activity of EvaD is relevant.

![Figure 1.18 Stereochemical inversions catalyzed by RmIC, NovW, and EvaD.](image-url)
RmlC, NovW and EvaD share high sequence identity (30-50%) and have been grouped together to form the tentatively named RmlC family of epimerases.\textsuperscript{55} While crystal structures have been reported for each epimerase, only limited mechanistic studies have been reported.\textsuperscript{57} As expected by the presence of the C-4'' ketone in all of the substrates and products a direct deprotonation/reprotonation mechanism has been implicated (Figure 1.2, p. 5). Evidence supporting the direct deprotonation/reprotonation mechanism is the observation of solvent (\textsuperscript{2}H\textsubscript{2}O) deuterium incorporation into substrate and product at the sites of epimerization.\textsuperscript{55-58} Furthermore, no cofactor is required for activity of any of the three enzymes, and no cofactor has been observed in the structures of the epimerases.\textsuperscript{59-61} Finally, examination of the X-ray crystal structures of the three epimerases reveals four conserved residues in the active sites: a histidine, an aspartate, a lysine, and a tyrosine. The histidine and aspartate are well poised to operate as a diad to remove the proton(s) from the substrate (structures on the left side of Figure 1.18). The lysine is in a position to stabilize negative charge at the C-4'' oxygen, and the tyrosine appears to be in a position to protonate the enolate intermediates generating epimeric product. A mutagenesis study of these four residues in RmlC from \textit{Streptococcus suis} is consistent with the proposed catalytic roles.\textsuperscript{59} The activities of H76N, K82A, and Y140F are all more than 1000-fold less than that of wild-type RmlC, and the $k_{cat}$ of D180A is ~100 fold less, while $K_m$ is unaffected.

Other sugar nucleotide epimerases operating at activated stereocentres are UDP-D-glucuronic acid 5-epimerase\textsuperscript{62} and CDP-3,6-dideoxy-D-glycero-D-glycero-4-hexulose 5-epimerase.\textsuperscript{63, 64} These cofactor independent epimerases are assumed to operate by the direct deprotonation/reprotonation mechanism, however detailed mechanistic studies have not yet been performed.
1.6.2 UDP-\textit{N}-acetylglucosamine 2-Epimerase (RffE)

UDP-\textit{N}-acetylglucosamine 2-epimerase (RffE) catalyzes the interconversion of UDP-GlcNAc and UDP-ManNAc (Figure 1.19). The epimerization occurs at an unactivated stereocentre, yet has been shown to have no requirement for a cofactor.\textsuperscript{65,66}

![Figure 1.19 Reaction catalyzed by UDP-\textit{N}-acetylglucosamine 2-epimerase (RffE).](image)

To explain this chemistry, it has been proposed that epimerization occurs via an \textit{anti}-elimination to generate 2-acetamidoglucal and UDP, followed by a \textit{syn}-addition to generate the epimeric product (Figure 1.20).\textsuperscript{65,66} In support of this mechanism it was observed that deuterium is incorporated at C-2" during epimerization in D\textsubscript{2}O. Upon extended incubation, the stable intermediates UDP and 2-acetamidoglucal were observed to accumulate in solution, indicating that occasionally (at 1/400\textsuperscript{th} the rate of epimerization) the intermediates are released from the active site.

![Figure 1.20 Mechanism of UDP-GlcNAc 2-epimerase (RffE).](image)
The most compelling evidence for this mechanism is the observation of a positional isotope exchange (PIX) of the oxygen bridging the sugar to UDP (Figure 1.21).\textsuperscript{65, 66} UDP-GlcNAc was synthesized with $^{18}$O at the anomic position, and it was observed that during epimerization the $^{18}$O scrambled to the two nonbridging positions on the $\beta$-phosphate of UDP. This is consistent with C-O bond cleavage occurring to generate 2-acetamidoglucal and UDP, and the $\beta$-phosphate having time to rotate such that any of the three chemically equivalent oxygen atoms could reform the C-O bond.

![Image of chemical structures](https://example.com/structure.png)

Figure 1.21 Positional isotope exchange (PIX) observed during epimerization by UDP-GlcNAc 2-epimerase (RffE).

RffE is an unusual example of a cofactor-independent epimerase that acts on an unactivated stereocentre. However, several key structural features permit this chemistry to occur, particularly the presence of a good leaving group in UDP, and the possibility of
oxocarbenium ion character in the transition state. While the C-2" position is unactivated in the sense that the C-H bond is not acidic, it is chemically activated in a broader sense.

1.7 SDR Sugar Nucleotide 4,6-Dehydratases

Sugar nucleotide 4,6-dehydratases catalyze the elimination of water to generate a 4-keto-6-deoxy product (Figure 1.22). This product can be further modified into a number of diverse sugar-nucleotides, and is an important intermediate in unusual sugar biosynthesis. There are three known sugar nucleotide 4,6-dehydratases, and all belong to the SDR enzyme family. This includes dTDP-glucose 4,6-dehydratase (RmIB), GDP-mannose 4,6-dehydratase (GMD), and CDP-glucose 4,6-dehydratase (CGD). All three dehydratases share common mechanistic features, and so to avoid repetition the mechanistic features common to all three dehydratases will be discussed first, and the particularities of each dehydratase will be discussed in following subsections.

![Figure 1.22 Generic sugar nucleotide 4,6-dehydratase catalyzed reaction.](image)

The accepted mechanism of dehydration of dTDP-glucose by RmIB is common to all three dehydratases (Figure 1.23). Oxidation at C-4" occurs first to generate a ketone intermediate and NADH. The ketone carbonyl acidifies the C-5" proton, enabling dehydration across the C-5"/C-6" bond to generate an α,β-unsaturated ketone. The hydride is then delivered to C-6" while C-5" is protonated. Consistent with this mechanism is the observation that protons from the medium (2H₂O, 3H₂O) are incorporated at C-5" of product.
One particularly informative experiment is the dehydration of substrate labelled with a deuterium at C-4" and a tritium at C-6". This experiment has been performed with RmlB (Figure 1.24), GMD, and CGD, and in each case the same result was observed. Upon dehydration, a chiral methyl group at C-6" of the product is generated, and it is possible to deduce which face the hydride is delivered to during the final reduction step. It was observed that incubation of dTDP-(6S)-[4-^2H,6-^3H]glucose with RmlB gives dTDP-4-keto-6-deoxy-(6R)-[6-^2H,6-^3H]glucose as the only product, and incubation of dTDP-(6R)-[4-^2H,6-^3H]glucose gives dTDP-4-keto-6-deoxy-(6S)-[6-^2H,6-^3H]glucose. The chirality of the methyl group was determined as follows. First, the product was subjected to Kuhn-Roth oxidation (CrO_3/H_2SO_4) to generate chiral acetate from the C-5" and C-6" portion of the sugar. The chiral acetate was then incorporated into malate stereospecifically, and the relative positions of the diastereotopic hydrogen atoms were determined using fumarase, which is known to exchange the pro-3R hydrogen with solvent protons. This experiment indicates: i) that the hydride transfer from C-4" to C-6" is strictly intramolecular, ii) the hydride transfer to C-6" is stereospecific, and iii) the replacement of the hydroxyl group at C-6" with a hydride occurs with inversion.
Figure 1.24 dTDP-glucose 4,6-dehydratase (RmlB) catalyzes a stereospecific hydride transfer from C-4" to C-6".

1.7.1 dTDP-glucose 4,6-Dehydratase (RmlB)

Of the three sugar nucleotide dehydratases, RmlB has been studied in the most detail. The fine details of the chemical mechanism reinforce the results of earlier studies. For example, the observation of the dTDP-4-keto-glucose-5,6-ene intermediate reinforces the mechanism described in Figure 1.23.\(^7^2\) This was possible via a rapid quench of the reaction with reductive stabilization of the intermediate using borohydride, to generate dTDP-hexopyranose-5,6-ene, which was observed via MALDI-TOF mass spectrometry. Mass spectrometry was also used to probe the dehydration step.\(^7^3\) Dehydration could occur in a stepwise manner via an enolate intermediate, or in a concerted fashion (Figure 1.25). To discern between these two possibilities the kinetics of the exchange of the C-5" proton with solvent hydrogen (\(^1^H/\(^3^H\)) and C-6" oxygen with solvent oxygen (\(^1^6^OH/\(^1^8^OH\)) were monitored using MALDI-TOF mass spectrometry. When dTDP-glucose-\(d_7\) was incubated with RmlB in \(^1^8^OH\) exchange of deuterium at C-5" and \(^1^8^O\) at C-6" were of found to occur at the same rate, indicating that exchange of these atoms
occurs via the dTDP-keto-glucose-5,6-ene intermediate. Thus deprotonation at C-\textit{5}'' does not occur independently (stepwise mechanism); the dehydration occurs in a concerted fashion.

Figure 1.25 Stepwise and concerted mechanisms for the dehydration step catalyzed by dTDP-glucose 4,6-dehydratase (RmlB).

Catalytic residues have been examined in detail for \textit{E. coli} RmlB. Sequence alignments and early X-ray crystal structures allowed identification of active site residues.\textsuperscript{74} The conserved triad of threonine 134, tyrosine 160 and lysine 164 has been studied by mutagenesis, and each residue is thought to play a role typical to SDR enzymes during the initial C-\textit{4}'' oxidation step.\textsuperscript{75} The residues which catalyze the dehydration step were identified using a combination of mutagenesis and mechanistic tools.\textsuperscript{76} To identify the acid catalyst which protonates the C-\textit{6}'' hydroxyl, the alternate substrate dTDP-6-deoxy-6-fluoroglucose, which eliminates HF instead of water, was used (Figure 1.26). Elimination of HF from dTDP-6-deoxy-6-fluoroglucose does not require protonation of the fluoride leaving group, and so it was found that mutants containing abasic residues in place of aspartate 135 did not dramatically affect HF elimination, but the same mutants slowed the normal dehydratase activity by two orders of magnitude. This demonstrates that aspartate 135 is the catalytic acid which protonates the C-\textit{6}'' hydroxyl during dehydration. To identify the catalytic base that deprotonates at C-\textit{5}'' during the dehydration,
solvent isotope exchange ($^{1}$H/$^{2}$H) into the C-5" position of the dTDP-4-keto-6-deoxyglucose product was used (Figure 1.27). Mutants containing abasic residues at glutamate 136 were approximately two orders of magnitude slower than wild-type RmlB at this exchange. This is consistent with glutamate 135 being the catalytic base for dehydration.

**Dehydration by D135A**

\[
\text{dTDP-glucose} \rightleftharpoons \text{Glu136} \rightleftharpoons \text{dTDP-4-keto-6-deoxyglucose}
\]

D135A $k_{\text{cat}} = 0.45 \%$ of WT $k_{\text{cat}}$

**Elimination of HF by D135A**

\[
\text{dTDP-6-deoxy-6-fluoroglucose} \rightleftharpoons \text{Glu136} \rightleftharpoons \text{dTDP-4-keto-6-deoxyglucose}
\]

D135A $k_{\text{cat}} = 67 \%$ of WT $k_{\text{cat}}$

Figure 1.26 The RmlB D135A mutant has a $k_{\text{cat}}$ for dehydration two orders smaller than wild-type (WT), yet $k_{\text{cat}}$ for elimination of HF is about equal to that of WT.

**Product isotope exchange with wild-type RmlB**

\[
\text{dTDP-4-ketoglucose-d}_7 \rightleftharpoons \text{Glu136} \rightleftharpoons \text{dTDP-4-ketoglucose-d}_5
\]

**Product isotope exchange with E136A mutant**

\[
\text{dTDP-4-ketoglucose-d}_7 \rightleftharpoons \text{Ala136} \rightleftharpoons \text{dTDP-4-ketoglucose-d}_6
\]

E136A $k_{\text{cat}} = 0.83 \%$ of WT $k_{\text{cat}}$

Figure 1.27 Product isotope exchange at C-5" with wild-type (WT) RmlB and E136A.
The roles that Rm1B tyrosine 160, aspartate 135 and glutamate 136 are thought to play are summarized in Figure 1.28. Tyrosine 160 deprotonates the C-4" hydroxyl during the oxidation step. In the concerted dehydration step glutamate 136 deprotonates at C-5" while aspartate donates a proton to the C-6" hydroxyl. In the final reduction step glutamate 136 donates a proton to C-5". In support of these assignments, the positions of these residues have been observed by X-ray crystallography and appear appropriate to their assigned roles.\textsuperscript{77, 78}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1_28.png}
\caption{Roles of catalytic residues of dTDP-glucose 4,6-dehydratase (Rm1B).}
\end{figure}

### 1.7.2 GDP-mannose 4,6-Dehydratase (GMD)

While GMD has not been subjected to rigorous mechanistic investigation it has been examined with X-ray crystallography and mutagenesis. The structure of the \textit{E. coli} GMD apoenzyme showed the conserved triad of serine, tyrosine and lysine are in positions analogous to other SDR enzymes, and that glutamate 135 (aligns with Glu136 of Rm1B) is in a position to catalyze acid/base chemistry in the active site.\textsuperscript{79} Conservative mutation of these residues gave mutants with $k_{\text{cat}}$ about three orders of magnitude lower than wild-type, while $K_m$ was relatively unaffected, suggesting these residues are important for catalysis. It is likely glutamate 135 is the
catalytic residue that deprotonates and reprotonates C-5" during the dehydration and reduction steps. In a crystal structures of GMD from *Arabidopsis thaliana* with NADPH and GDP-rhamnose bound,\textsuperscript{80} and from *Pseudomonas aeruginosa* with NADPH and GDP bound,\textsuperscript{81} the corresponding glutamates are in a similar position that is consistent with the same glutamate being the acid/base deprotonating C-5" during dehydration. However, all three of these crystal structures lack a traditional acid/base residue in the position corresponding to that of aspartate 135 of *E. coli* RmlB. Therefore, the catalytic residue which protonates the C-6" hydroxyl during dehydration has been suggested to be played by either a) the same glutamate that deprotonates at C-5" (Glu135 of *E. coli* GMD)(Figure 1.29), or b) a suitably positioned serine (Ser133 of *E. coli* GMD).\textsuperscript{80,81}

![Figure 1.29 Possible dual catalytic role of glutamate 135 of *E. coli* GMD.](image)

**1.7.3 CDP-glucose 4,6-Dehydratase (CGD)**

CGD has been studied by X-ray crystallography, but active site residues have not been the subject of rigorous mechanistic study. CGD and has been successfully labeled with a suicide inhibitor which is believed to label an active site nucleophile.\textsuperscript{82} Unfortunately, the labeled residue was not identified. The X-ray crystallographic data is the most informative study of active site residues to date.

A crystal structure of CGD from *Yersinia pseudotuberculosis* with NAD\textsuperscript{+} bound has been solved to 1.8 Å.\textsuperscript{83} The positions of the conserved triad of serine, tyrosine and lysine are similar to those of other SDR enzymes, and similar catalytic roles have been proposed. The
residue responsible for C-5" deprotonation/reprotonation during dehydration and reductive steps appears to be lysine 134, due to its appropriate position in the active site. The identity of the active site acid residue that protonates the C-6" hydroxyl is not clear, though aspartate 135 is in the vicinity. It should be emphasized that no substrate is bound to this structure. In a subsequent crystal structure of CGD from *Salmonella typhi* with cofactor and CDP-D-xylose bound the positions of the active site residues during catalysis may be better approximated. In this structure the corresponding aspartate (Asp136 in this organism) is in a similar position as the known catalytic acid in RmlB (Asp135, Figure 1.28), and appears to be the catalytic acid that protonates the C-6" hydroxyl in CGD.

1.8 Proposed Research

1.8.1 ADP-\(L\)-glycero-\(D\)-manno-heptose 6-Epimerase (HldD)

ADP-\(L\)-glycero-\(D\)-manno-heptose 6-epimerase (HldD) is a member of the SDR enzyme family that catalyzes the interconversion of ADP-\(D\)-glycero-\(D\)-manno-heptose (ADP-\(D,D\)-Hep) and ADP-\(L\)-glycero-\(D\)-manno-heptose (ADP-\(L,D\)-Hep) (Figure 1.30).\(^5\) ADP-\(L,D\)-Hep serves as the activated form of \(L\)-glycero-\(D\)-manno-heptose (\(L,D\)-Hep) which is incorporated into lipopolysaccharide (LPS) in Gram-negative bacteria.\(^{37,85,86}\)

```
\[\text{ADP-}\(D,D\)-Hep} \xrightarrow{\text{HldD}} \text{ADP-}\(L,D\)-Hep\]
```

Figure 1.30 Reaction catalyzed by ADP-\(L\)-glycero-\(D\)-manno-heptose 6-epimerase (HldD).

\(^5\) The naming of the unusual heptose moiety of these sugar nucleotides (\(D\)-glycero-\(D\)-manno-heptose and \(L\)-glycero-\(D\)-manno-heptose) is based upon IUPAC nomenclature. The stereochemical configurations of C-2", C-3", C-4" and C-5" are described by the \(D\)-manno prefix, and the C-6" stereochemical configuration is described by the \(D\)- or \(L\)-glycero prefix.
LPS is a major component of the outer surface of the outer membrane of Gram-negative bacteria. Of the three distinct regions of LPS (lipid A, the core region, and the O-antigen polymer), L,D-Hep is found in the inner core, which also contains the unusual sugar 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo)(Figure 1.31). While the structure of LPS varies among organisms, the L,D-Hep unit is a generally conserved feature. It has been found that mutant strains of *E. coli* and *Salmonella typhimurium* lacking HldD activity are viable, but have a truncated LPS and an increased sensitivity to a number of hydrophobic antibiotics. Minimum inhibitory concentrations of various antibiotics were found to decrease 30-64 fold for *E. coli*, and 16-256 fold for *S. typhimurium*, versus wild-type bacteria.

![Diagram of E. coli K-12 LPS](image)

Figure 1.31 Structure of *E. coli* K-12 LPS, the major component of the outer surface of the outer membrane of Gram-negative bacteria.

HldD is one enzyme of several catalyzing the biosynthesis of ADP-L,D-Hep from sedoheptulose 7-phosphate (Figure 1.32). Sedoheptulose-7-phosphate isomerase (GmhA) generates the aldose D-glycero-D-mannoheptose 7-phosphate (D,D-Hep 7P), which is the substrate for the kinase activity of the bifunctional enzyme D-glycero-D-mannoheptose 7-phosphate kinase/D-glycero-β-D-mannoheptose 1-phosphate adenylyltransferase (HldE). The kinase activity of HldE installs a phosphate at the anomeric position with β-configuration. This β-anomeric linkage is unusual for sugar nucleotides, which are mostly α-linked. The D-glycero-
β-D-mannoheptose 1,7-bisphosphate (D,D-Hep 1,7-diP) is then subjected to the activity of D-glycero-α,β-D-mannoheptose 1,7-bisphosphate phosphatase (GmhB), which cleaves the C-7 phosphate to generate D-glycero-β-D-mannoheptose 1-phosphate (D,D-Hep 1P). The adenyllyltransferase activity of HldE completes the nucleotide portion of the sugar nucleotide, generating ADP-D,D-Hep. HldD completes the biosynthesis of ADP-L,D-Hep via epimerization at C-6", and various transferases incorporate the L,D-Hep sugar into LPS.

Figure 1.32 Biosynthesis of ADP-L,D-Hep from sedoheptulose 7-phosphate.85

While there has been little mechanistic study of HldD, the available clues suggest a mechanism involving transient oxidation. Observations of the available crystal structure confirm that HldD is a member of the SDR enzyme family, and that it binds one equivalent of NADP⁺ per subunit of enzyme.90 This is consistent with observations that HldD tightly binds its NADP(H)⁺ cofactor, and that it is required for activity.91 It is likely that HldD uses its cofactor to transiently oxidize the substrate during catalysis, as the C-6" stereocentre that undergoes
inversion is unactivated. This is supported by chemical logic, which predicts that a direct deprotonation/reprotonation mechanism cannot occur at the unactivated C-6' stereocentre (Figure 1.30), while a redox mechanism could explain how this epimerization occurs.\(^3\) In the crystal structure, NADP\(^+\) is in a suitable position for redox chemistry, however the site of oxidation on the sugar moiety is not clear.\(^90\) This is not surprising, as the substrate analog bound in the active site of HldD is ADP-\(\alpha\)-D-glucose, which would not bind in the active site in an identical manner as the natural substrate since it has the wrong stereochemistry at C-1' and C-2', and is missing one hydroxymethylene group at C-6'. While the ADP moiety is well resolved in the 2.0 Å structure, the sugar moiety cannot be resolved in seven of the ten active sites in the asymmetric unit. In the three active sites where the glucose moiety can be resolved, the glucose sits in two different orientations that differ significantly.

The mechanism of HldD must necessarily be unique amongst sugar nucleotide epimerases. The epimerized stereocentre is at a unique C-6' position where no other sugar nucleotide epimerase operates. The mechanistic clues suggest that HldD operates by transient oxidation, but the position of oxidation is not obvious. There are three reasonable positions on the sugar moiety of the sugar nucleotide where transient oxidation would enable epimerization. One scenario is that the C-4' position is transiently oxidized. Oxidation at C-4' generates a ketone intermediate which could undergo dehydration across the C-5'/C-6' bond (dehydration mechanism, Figure 1.33). Water then adds to the opposite face of the \(\alpha,\beta\) unsaturated ketone and the C-4' carbonyl is reduced to generate the epimeric product. Considering the similarity in each of these steps with those of the 4,6-dehydratases, this mechanism seems quite likely. Alternatively, transient oxidation could occur at C-6'. The C-6' ketone could be reduced from the opposite face to generate the epimeric product (direct redox mechanism, Figure 1.33). The third possibility is that HldD oxidizes at C-7' to generate an aldehyde (C-7' redox mechanism,
Figure 1.33. The carbonyl acidifies the adjacent proton, allowing deprotonation to generate a resonance stabilized carbanion that may be protonated from the opposite face. Reduction at C-7" generates the epimeric product.

**Dehydration Mechanism**

**Direct Redox Mechanism**

**C-7" Redox Mechanism**

Figure 1.33 Possible mechanisms of HldD.
1.8.2 UDP-N-acetylglucosamine 5-Inverting 4,6-Dehydratase (PseB)

UDP-N-acetylglucosamine 5-inverting 4,6-dehydratase (PseB) is a recent and unique addition to the SDR enzyme family. PseB from *Campylobacter jejuni* and *Helicobacter pylori* has been demonstrated to convert UDP-N-acetylglucosamine (UDP-GlcNAc) to UDP-2-acetyl-2,6-dideoxy-β-L-arabino-4-hexulose (UDP-arabino-sugar) (Figure 1.34). This is the first example of a sugar nucleotide 4,6-dehydratase that inverts the configuration at C-5" during catalysis.

Figure 1.34 Reaction catalysed by PseB.

PseB catalyses the first step in the biosynthesis of CMP-pseudaminic acid (Figure 1.35). Pseudaminic acid is a nine carbon α-keto acid, or sialic acid, found to post-translationally modify flagellin (the protein which assembles to form flagella) in *C. jejuni* and *H. pylori*. The presence of pseudaminic acid has been found to be crucial for the assembly of functional flagella, and is therefore required for bacterial motility and invasion of the host intestinal tract. As *C. jejuni* and *H. pylori* are pathogenic bacteria known to cause duodemic ulcers and gastrointestinal diarrhea, respectively, an understanding of the biosynthetic machinery essential for the viability of these pathogens is of interest for human health reasons. The biosynthesis of pseudaminic acid starts with the action of PseB on UDP-GlcNAc, which generates the UDP-arabino-sugar, the substrate for the PLP-dependent aminotransferase PseC. PseC installs an amino group at C-4", which is subsequently acetylated by the action of N-acetyltransferase PseH. The hydrolase PseG then cleaves the UDP from the sugar.
nucleotide,\textsuperscript{100} and pseudaminic acid synthase PseI mediates the condensation of the free sugar with PEP to generate pseudaminic acid.\textsuperscript{101} Finally, CMP-pseudaminic acid synthetase PseF installs the CMP group at C-2 to generate the activated form of the sugar.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Biosynthesis of CMP-pseudaminic acid from UDP-N-acetylglucosamine.}
\end{figure}

There has been little mechanistic study of PseB, although PseB from \textit{H. pylori} has been studied by X-ray crystallography and a mechanism has been proposed.\textsuperscript{102} These studies show that NADP\textsuperscript{+}/NADPH is tightly bound to the active site without exogenous cofactor added, indicating that the cofactor is co-purified with PseB. The active site was found to contain the conserved SDR triad of threonine, tyrosine and lysine, and it is thought that these residues play similar roles as in other SDR enzymes. Mutagenesis of the tyrosine (Y141F) destroys catalytic activity, consistent with its role as catalytic base during oxidation.\textsuperscript{103} Other residues in the active site include aspartate 132 and lysine 133, which are in appropriate positions to catalyze the dehydration step.\textsuperscript{102} Consistent with this, mutation of the lysine (K133M and K133E) results
in a loss of activity. The authors calculated the pKₐ of each putative catalytic residue, and proposed a catalytic mechanism (Figure 1.36). Tyrosine 141 is proposed to deprotonate the C-4" hydroxyl while NADP⁺ accepts the C-4" hydride during the initial oxidation step. In the dehydration step lysine 133 plays a dual role of acid and base, while aspartate 132 serves to 'increase the nucleophilic character of the C-6" hydroxyl'. Lysine 133 abstracts the proton from C-5" concertedly with the abstraction of a proton from the lysine by the C-6" oxygen, to generate water and the α,β unsaturated ketone. In the final reduction step it is proposed that the hydride is delivered to the C-5" carbon as the newly generated water molecule donates a proton to C-6".

Figure 1.36  Literature proposed mechanism of PseB.¹⁰²
Given the precedent of the three dehydratases discussed earlier in this chapter, RmlB, GMD and CGD, the above literature proposed mechanism for PseB is unlikely. While the initial oxidation is likely to occur as proposed, the dehydration step seems needlessly complicated, and the reduction step is extremely unlikely. In the alternate mechanism proposed here, lysine 133 performs only one function during dehydration, to deprotonate C-5'' (Figure 1.37). It seems unlikely that an aspartate that is within hydrogen bonding distance of the C-6'' hydroxyl would not participate in the reaction and therefore aspartate 132 is proposed to act as the catalytic acid during dehydration. In the reduction step, the literature proposal that the hydride is delivered to C-5'' is without precedent and in defiance to chemical logic. It is proposed here that the hydride is delivered to C-6'', and a proton is delivered to C-5''. The identity of the catalytic residue delivering the proton in the reduction step is difficult to predict.

There must be movement of the α,β unsaturated ketone intermediate and/or enzyme in order to allow NADPH appropriate access to C-6''. Any of the three residues are candidates for this role, however lysine 133 and tyrosine 141 are favored, given they should be in the appropriate protonation states.

Figure 1.37 Proposed mechanism of PseB.
1.8.3 Aims of this Thesis

The aims of this thesis are to address the mechanisms of ADP-\(L\)-glycero-D-manno-heptose 6-epimerase (HldD) and UDP-\(N\)-acetylglucosamine 5-inverting 4,6-dehydratase (PseB). HldD is a unique epimerase, and several reasonable mechanistic possibilities can be envisioned. There is no other example of a sugar nucleotide epimerase that operates at C-6\(^\prime\), and as such the operative mechanism must necessarily be unique amongst this class of enzymes. The first goal is to establish the chemical mechanism for the epimerization. Following this, identification of catalytic residues will be addressed. PseB is also unique amongst sugar nucleotide dehydratases as it is the only dehydratase that inverts at C-5\(^\prime\) during dehydration. How this is achieved must be elucidated in the interest of understanding this class of enzymes more fully. To this end, the literature proposed mechanism needs to be probed with the aim of establishing an evidence based mechanism. With the establishment of the chemical mechanism, the identity and roles of the catalytic residues in PseB will be sought out.

In addition to the mechanistic intrigue that HldD and PseB hold, there is a human health interest in studying these enzymes. Studies have indicated that both these enzymes are important to the viability of the relevant bacteria. Both HldD and PseB are unique to bacteria, and as such they are potentially suitable drug targets. A mechanistic understanding will allow insight into the design of inhibitors.

During the course of this research several other individuals in this laboratory were involved with the study of HldD and PseB. As their work directly influenced the work described in this thesis, their work will be described in a relevant fashion throughout this thesis. To avoid confusion the work of others will be clearly denoted as such. This includes the work of Dr. Jay Read (HldD), Dr. Alain Mayer (HldD), Dr. Raef Ahmed (HldD), and Jackie Bassiri (PseB).
Chapter Two

The Chemical Mechanism of ADP-L-glycero-D-manno-heptose 6-Epimerase
2.1 Introduction

The focus of this chapter is the elucidation of the chemical mechanism of HldD. As discussed in chapter one, HldD is likely to operate by a redox mechanism and it would be reasonable to expect that transient oxidation could occur at C-4", C-6" or C-7" (Figure 1.33, p. 36). The deduction of the site of transient oxidation was the first goal of the project, which was also being pursued by fellow lab member Dr. Jay Read. The next goal of the project was to provide direct evidence that supports the existence of the postulated intermediates in the favoured reaction mechanism. A major tool that was employed involved the design and testing of rationally designed substrate/intermediate analogs. As this strategy was executed simultaneously with other research in the lab, relevant details of the work of others will be discussed in a timely fashion.

2.2 ADP-6-deoxy-6-fluoro-\(\beta\)-d-mannose as a Mechanistic Probe

Most SDR enzymes operating on sugar nucleotides catalyze redox chemistry at C-4", and therefore the dehydration mechanism was initially favoured for the HldD reaction (Figure 1.33, p. 36). Of the eight other SDR enzymes discussed in Chapter One, seven are known to catalyze redox chemistry at C-4". In particular, HldD shares many structural features with GalE (UDP-galactose 4-epimerase). The sequences of the \textit{E. coli} HldD and GalE are 23 \% identical and the tertiary structures are remarkably similar.\(^9\) These commonalities suggested that HldD might also share C-4" redox chemistry in its mechanism of epimerization.

To probe the ability of HldD to catalyze C-4" redox chemistry the fluorinated substrate analog ADP-6-deoxy-6-fluoro-\(\beta\)-d-mannose 1 was designed (Figure 2.38). Several scenarios for 1 can be imagined, assuming HldD accepts this substrate analog and operates by C-4" redox chemistry. Upon oxidation at C-4" an irreversible elimination of HF would generate an \(\alpha,\beta\)
unsaturated ketone. The fate of this α,β unsaturated ketone could be one of the following: nucleophilic 1,4 addition by an active site nucleophilic residue, hydration of the alkene and reduction to ultimately give ADP-mannose, or release from the active site. Observation of any of these scenarios would be evidence for a C-4″ oxidation mechanism. In all these scenarios it was anticipated that the C-7″ moiety is non-crucial for catalysis, and so a six-carbon sugar moiety has been chosen for this mechanistic probe due to synthetic considerations. The chemo-enzymatic synthesis of compound 1, as well as the cloning of a synthetically useful enzyme HldE (see below), became the first goals of this thesis.

Figure 2.38 Possible reactivity of substrate analog 1 in the active site of HldD.

2.2.1 Cloning of D-Glycero-D-mannoheptose 7-Phosphate Kinase/ D-Glycero-β-D-mannoheptose 1-Phosphate Adenylyltransferase (HldE)

Mechanistic studies of HldD are complicated by the fact that the substrate is not commercially available, is difficult to make, and has limited stability. The most complicating feature of these sugar nucleotides is the β-mannosyl linkage, which is notoriously difficult to synthesize and places the C-2″ hydroxyl on the same side of the ring as the ADP moiety, which enables a ring closure and loss of AMP to occur readily under mildly basic conditions (Figure 2.39).\textsuperscript{104} Under mildly acidic conditions the substrate can also undergo hydrolysis (loss of ADP) via oxocarbenium intermediates.
As the β-mannosyl linkage is known to be problematic, a method to generate ADP-β-
mannosyl sugars that allows for expeditious purification with minimal handling was sought. To 
this end a recombinant source of the biosynthetic enzyme D-glycero-D-mannoheptose 7-
phosphate kinase/D-glycero-β-D-mannoheptose 1-phosphate adenylyltransferase (HldE, Figure 
1.32, p. 34) was made available. The second function of HldE transfers an adenylyl group onto 
D-glycero-β-D-mannoheptose 1-phosphate (D,D-Hep 1P) to generate ADP-D-glycero-D-
mannoheptose (ADP-D,D-Hep)(Figure 2.40). By using the adenylyltransferase activity of HldE in the 
final step of the synthesis of ADP-D,D-Hep, the problematic moiety is installed last and 
minimal handling of ADP-D,D-Hep is required. By coupling HldE with inorganic 
pyrophosphatase (which hydrolyses pyrophosphate to phosphate) the reaction can be driven to 
high yields, facilitating purification.

The preparation of recombinant His-tagged HldE was completed using a Novagen Xa 
ligation-independent cloning (LIC) kit, briefly described here. The gene hldE was amplified
from genomic E. coli K-12 W3110 DNA by PCR to create a double-stranded piece of DNA containing \textit{hldE} bracketed by two designed oligomers 15 and 17 nucleotides long. The two strands in the PCR product are perfectly matched and termed 'blunt ended'. The PCR product was then incubated with T4 DNA polymerase and dGTP. T4 DNA polymerase has 3'→5' exonuclease activity as well as 5'→3' polymerase activity, and under these conditions T4 polymerase hydrolyzes nucleotides from the 3'-ends of the PCR product until the first guanosine is reached. The DNA strands are no longer perfectly matched; they contain 12- or 15-base single stranded overhangs. These overhangs are complementary to the overhangs in a commercially available pET-30 vector, such that the target insert (containing \textit{hldE}) anneals with the vector to give a double nicked plasmid. The annealing mixture is transformed directly into Novablu GigaSingles competent cells, where ligation occurs to form a plasmid. The plasmid contains a gene which encodes kanamycin resistance, allowing selection of only those cells containing the plasmid with the \textit{hldE} gene. At this point the plasmid was purified and \textit{hldE} was sequenced at the Nuclei Acid Protein Service (NAPS) unit at UBC to verify that no errors were introduced during the above process. The plasmid was then transformed into E. coli JM109(DE3) competent cells for protein overexpression. The cells were grown in kanamycin containing media at 37 °C to an OD\textsubscript{600} ~0.6-0.9 at which time IPTG (isopropyl \(\beta\)-D-1-thiogalactopyranoside) was added to induce overexpression of \textit{hldE}. IPTG is able to induce overexpression of \textit{hldE} because there is a \textit{lac} operon upstream from \textit{hldE}. Normally, in the absence of lactose or IPTG, a repressor binds to the \textit{lac} operon, preventing the T7 RNA polymerase from binding to a T7 promoter region on the plasmid. IPTG binds to the repressor, causing the repressor to release from the \textit{lac} operon, allowing T7 RNA polymerase to bind and express \textit{hldE}. Overexpression is allowed to occur for five hours, at which time OD\textsubscript{600} ~1.6-1.8. The cells were then harvested, lysed, and the soluble fraction was loaded onto an affinity
chromatography column containing immobilized nickel (Ni$^{2+}$). The hexahistidine tag on HldE binds with Ni$^{2+}$ allowing the cell lysate to be washed from the column. HldE was eluted with 500 mM imidazole, and the HldE containing fractions were dialyzed into a storage buffer containing 10% glycerol. HldE in this buffer was then flash frozen in liquid nitrogen and stored at $-70^\circ$C for up to 12 months without significant loss of activity. HldE prepared in this manner typically yields ~20 mg per liter of culture. SDS-PAGE of the purified protein revealed a single band at ~56 kDa, consistent with the predicted molecular weight and indicating greater than 90% purity (Figure 2.41).

![SDS-PAGE](image)

Figure 2.41 Overexpression and purification of HldE followed with SDS-PAGE. Lanes: 1 and 10) molecular weight standards BSA (29 kDa), ovalbumin (49 kDa) and carbonic anhydrase (66 kDa). 2) Cell lysate at OD$_{600}$ = 0.6 (before IPTG). 3) 2 h after inducing. 4) Crude cell lysate after 5 h, OD$_{600}$ = 1.6. 5) Ni$^{2+}$ column flow through (5 mM imidazole). 6) HldE containing fraction (500 mM imidazole). 7-9) Next three fractions (500 mM imidazole).

### 2.2.2 Synthesis of ADP-6-deoxy-6-fluoro-β-D-mannose 1

ADP-6-deoxy-6-fluoro-β-D-mannose 1 was made in ten steps from α-methyl mannopyranoside (Figure 2.42). The first key step is the introduction of the fluorine group at C-6 which was accomplished with DAST (diethylaminosulfur trifluoride), which directly displaces a hydroxyl with fluorine. DAST is relatively mild and can be used with acid sensitive compounds, and as such is a common fluorinating reagent in carbohydrate chemistry. Normally, DAST is selective for the less hindered alcohol, and has been used to monofluorinate
primary alcohols in the presence of secondary alcohols in acceptable yields.\textsuperscript{105} In the case of $\alpha$-methyl mannopyranoside, however, it has been found that fluorination occurs at C-6 and C-4 equally as well, and it was not possible to obtain the C-6 monofluorinated product in this manner.\textsuperscript{106, 107} This is in contrast to $\alpha$-methyl glucopyranoside, which undergoes C-6 monofluorination under similar conditions.\textsuperscript{105} It has been proposed that the axial C-2 hydroxyl of $\alpha$-methyl mannopyranoside assists the C-4 fluorination in an ancillary manner.\textsuperscript{106} To avoid this problem a C-4 protecting group was used in the synthesis of 1. A diacetal protecting group was chosen for this purpose due to its facile installation and the fact that it can be later deprotected under the same conditions as the acetal at C-1. Thus the known 3,4-diacetal \textsuperscript{2} was prepared,\textsuperscript{108} and treatment with DAST gave 3 in 21% yield. It should be pointed out that low yields are commonplace in similar DAST reactions.\textsuperscript{105} In an attempt to improve the fluorination yield a benzyl protecting group was installed at C-2 of compound 2 under normal benzylation conditions (sodium hydride, one equivalent of benzyl bromide in dimethyl formamide at zero degrees Celsius) to give the known compound (2'S,3'S)-methyl 2-O-benzyl-3,4-O-(2',3'-dimethoxybutane-2',3'-diyl)-$\alpha$-D-mannopyranoside.\textsuperscript{109} Selective benzylation of the C-2 hydroxyl in the presence of a primary alcohol under these conditions is known for a similar mannosyl compound, however the cause of the selectivity is not understood.\textsuperscript{110} Fluorination of this derivative gave the C-6 fluorinated product in 30% yield, a slight improvement over the fluorination of 2, however the additional benzylation (50% yield) and hydrogenation (90% yield) steps lowered the overall yield from 2 to 3, making the circuitous route unattractive. With the fluorine installed the protecting groups were removed from compound 3 and the sugar was peracetylated to give 4, which was then anomerically deacetylated to give 5.
Figure 2.42 Synthesis of ADP-6-fluoro-6-deoxy-D-mannose 1. a) 2,3-butanedione, trimethyl formate, CSA, MeOH, reflux. b) DAST, CH₂Cl₂, −40 °C. c) TFA, CHCl₃, RT. d) Ac₂O, H₂SO₄. e) HNMe₂, MeCN. f) dibenzyl N,N-diisopropylphosphoramidite, 1,2,4-triazole, CH₂Cl₂. g) 30 % H₂O₂, THF, −78 °C. h) H₂, Pd/C, MeOH, NEt₃. i) 2:3:0.6 MeOH/0.1M triethylammonium bicarbonate/NEt₃, −20 °C, 4 days. j) ATP, HldE, inorganic pyrophosphatase, 37 °C.

Installation of the β-phosphate was achieved using a phosphoramidite reagent. This reagent is known to give mixtures of α- and β-anomers, generally favoring the more stable α-anomer.¹¹¹ This proved to be the case in the phosphoramidite reaction with 5, however a significant portion (18 % yield) of the β-anomer was obtained. Anomers of mannose are indistinguishable by measurement of proton-proton coupling constants (J values) due to weak H1-H2 coupling observed in both anomers. Therefore a NOE (nuclear Overhauser effect) was measured to distinguish between the anomers. NOE is a through-space effect that enhances the intensity of signals within 5 Å of the selectively irradiated signal.¹¹² No J coupling need to be present between the two nuclei. The phenomenon arises from dipole-dipole interactions, and
when one of the nuclei is irradiated the Boltzmann population distribution of the other nucleus is altered, enhancing the intensity of its resonance. For protons the maximum enhancement is 50%. In the case of 6 the differences between the proximity of H1 to H3 and H5 is dramatic between the α- and β-anomers (Figure 2.43). As the equatorial H1 is further removed from H5 in the α-anomer, no significant NOE between H1 and H5 was observed. The axial H1 of the β-anomer is closer to H5, and as such a strong NOE is observed between H1 and H5.

Figure 2.43 Differentiation of α- and β-anomers of 6 by NOE difference spectroscopy. A) bottom: partial $^1$H NMR spectrum of the α-anomer of 6. Top: NOE difference spectrum with irradiation at 4.00 ppm. B) Bottom: partial $^1$H NMR spectrum of the β-anomer of 6. Top: NOE difference spectrum irradiating at 3.73 ppm (all spectra 300 MHz, CDCl$_3$).

The synthesis of 1 was completed with the assistance of HldE (Figure 2.42). The protecting groups were first removed from 6 to give the fully deprotected sugar 7. As anticipated, HldE accepted 7, and the adenylyltransferase activity generated the final product 1.
2.2.3 Incubation of ADP-6-deoxy-6-fluoro-β-D-mannose 1 with HldD

The source of the epimerase HldD studied in this thesis is a recombinant protein bearing an N-terminal His-tag. A plasmid was made available by Dr. Jay Read, who isolated the hldD gene from *E. coli* K-12 W3110 and inserted it into a pET-30 vector (Novagen). This is the same vector that was used for the production of HldE. This plasmid was transformed into JM109(DE3) cells, and following overexpression, the resulting HldD was purified and stored in a similar manner as HldE. SDS-PAGE of affinity-purified HldD reveals one protein band at ~40 kDa, indicating the epimerase is isolated in at least 90% purity (Figure 2.44).

The incubation of 1 with HldD was monitored by $^1$H and $^{19}$F NMR spectroscopy. At this time Dr. Jay Read and Dr. Raef Ahmed had successfully synthesized the two substrates for HldD, ADP-D,D-Hep and ADP-L,D-Hep and had observed the epimerization reaction by $^1$H NMR spectroscopy. Therefore, epimerization of ADP-D,D-Hep was used as a control in these experiments to ensure that HldD was active under the experimental conditions. Unfortunately even upon extended incubation of 1 with HldD (48 h) no spectral changes were observed in these experiments, while epimerization of ADP-D,D-Hep occurred quickly (within 5 min). This shows that 1 is not an alternative substrate for HldD. These observations are in agreement with

![Figure 2.44 Purification of HldD followed by SDS-PAGE. CL = crude cell lysate, FT = flow through nickel affinity column. CW = column wash with 100 mM imidazole. Lanes 1 and 2 = HldD containing fractions eluted with 500mM imidazole. MW = molecular weight standards BSA (29 kDa) and carbonic anhydrase (66 kDa).](image-url)
other results that were generated simultaneously in the lab. Those studies, discussed in more
detail below, indicate that HldD does not operate by a C-4'' redox mechanism. The reactivity of
1 was not pursued further.

2.2.4 Other Work in the Lab

By this time the results of the work of fellow lab member Dr. Jay Read were becoming
available. Dr. Read had gathered evidence that eliminated the dehydration and C-7'' redox
mechanisms (Figure 1.33, p. 36). Dr. Read found that neither deuterium nor solvent-derived
oxygen was incorporated into the substrates during catalysis (in D₂O or ¹⁸OH₂) as verified by
¹H NMR spectroscopy and mass spectrometry. This argues against the dehydration and C-7''
redox mechanisms, as both mechanisms would be expected to incorporate solvent-derived
deuterium, and the dehydration mechanism would be expected to incorporate solvent-derived
¹⁸O. It is possible that the active site of HldD is extremely well controlled, such that only
protons or oxygen atoms derived from the substrate are returned to the intermediate during
catalysis. However, Dr. Read also found that both a 4''-deoxy analog and a 7''-deoxy analog of
ADP-D,D-Hep are proficient substrates for HldD (Figure 2.45). This eliminates any mechanism
involving transient C-4'' or C-7'' oxidation, and the only reasonable mechanism is the direct
redox mechanism (Figure 1.33, p. 36).
Figure 2.45 Observation that the 4-deoxy and the 7-deoxy substrate analogs are proficient substrates for HldD argues strongly against mechanisms involving C-4" and C-7" transient oxidation. Findings of Dr. Jay Read.

2.3 ADP-β-D-manno-hexodialdose as a Mechanistic Probe

With the elimination of mechanisms involving C-4" and C-7" transient oxidation, the favoured mechanism became the direct redox mechanism (Figure 2.46, top). Until this point, only indirect evidence suggested that HldD operates by the direct redox mechanism, and so establishing the mechanism of HldD continued to be the goal in the project. One possibility was to synthesize the putative ketone intermediate, and determine if the enzyme would be able to recognize it as a catalytically competent species (Figure 2.46, bottom).
Direct Redox Mechanism

Test for Catalytic Competence of Intermediate

This strategy is complicated by several factors. The first problem is that this experiment requires the tightly bound cofactor to be in the reduced state (NADPH). Normally the cofactor isolated with HldD is mostly in the oxidized form. As the cofactor is tightly bound and does not readily exchange into solution, HldD must be denatured to exchange NADP\(^+\) with NADPH, or reduced in situ with a reducing agent, such as sodium borohydride. Reducing the cofactor in situ presents a problem with removing residual reducing agent, as it would react with the ketone intermediate, giving a false positive result. The other problem with the experiment is that reduced HldD (with NADPH bound) is required in stoichiometric amounts. This is particularly problematic due to the fact that the assay of choice is \(^1\)H NMR spectroscopy, which requires millimolar concentrations. As HldD has a molecular weight of \(~40\) kDa, a single experiment would require about 20 mg of enzyme, which may not be soluble in such a small volume, and would have to be removed prior to measuring the spectrum. In addition, the above experiment requires synthesis of the ketone intermediate, which is nontrivial. To reiterate, the experiment described in Figure 2.46, while not impossible, is complicated on several levels.
An easier experiment to perform that would yield essentially the same information is to incubate ADP-β-D-manno-hexodialdose 8 with catalytic amounts of native HldD (Figure 2.47). It was anticipated that the aldehyde 8 can be either oxidized or reduced and therefore a dismutation of 8 with catalytic amounts of HldD would occur. An aldehyde is in rapid equilibrium with its hydrated form in aqueous solution. The unhydrated form of 8 resembles the ketone intermediate and should be able to accept a hydride from NADPH to give ADP-mannose 9. The hydrated form of 8 is not unlike the natural substrates and could lose a hydride to NADP+ to form ADP-mannuronic acid 10. With catalytic amounts of HldD, one would expect a redox balanced dismutation to occur provided the C-7" hydroxymethylene of the natural substrate is non-crucial for activity. Since both the oxidation and reduction half-reactions are expected to be thermodynamically favourable the process should go to completion. Observation of dismutation would provide evidence that HldD is able to both oxidize and reduce at C-6", providing positive evidence for the direct redox mechanism.

![Diagram](image)

Figure 2.47 Proposed strategy to probe the direct redox mechanism: dismutation of ADP-β-D-manno-hexodialdose 8.
2.3.1 Synthesis of ADP-β-D-manno-hexodialdose 8

The strategy used to prepare 8 is based on a previous synthesis of UDP-α-D-gluco-hexodialdose completed by Dr. Robert Campbell, a former graduate student in the Tanner lab. As an aldehyde is somewhat reactive, the plan was to form the C-6 aldehyde functionality at an early step, then to mask the aldehyde as an alkene (Figure 2.48). The acetate protecting group was chosen in this synthesis as its effectiveness and compatibility had been proven in the synthesis of UDP-α-D-gluco-hexodialdose and ADP-6-deoxy-6-fluoro-β-D-mannose. Thus, the known compound 11 was prepared in two steps from mannose, with selective crystallization yielding the pure β-anomer. From 11, a Pfitzner-Moffatt oxidation (or Moffatt oxidation) generated the C-6 aldehyde, and the Lombardo reaction yielded alkene 12. These are mild conditions that avoid use of base and therefore do not promote the facile β-elimination of acetic acid from compound 11. The activating agent CMC used in the Moffatt oxidation is a water-soluble version of the more common DCC. It is used here because the urea byproduct derived from it is readily removed upon extraction during work up. The olefination step is carried out without purification of the unstable aldehyde to yield the stable alkene 12, in 12 % yield from 11. This is the same yield Dr. Campbell reported in the synthesis of the gluco-analog of 12.
Figure 2.48 Synthesis of ADP-β-D-manno-hexodialdose 8. a) TrCl, pyr., then Ac₂O. b) HBr, acetic acid, 45 s. c) CMC, DMSO, Cl₂HCCOOH. d) CH₂Br₂, Zn, TiCl₄. e) HNMe₂, MeCN. f) dibenzyl N,N-diisopropylphosphoramidite, 1,2,4-triazole, CH₂Cl₂. g) 30% H₂O₂, THF, –78 °C. h) O₃, –78 °C, then Me₂S. i) H₂, Pd/C, MeOH, NEt₃. j) 2:3:0.6 MeOH/0.1M triethylammonium bicarbonate/NEt₃, –20 °C, 4 days. k) ATP, HldE, inorganic pyrophosphatase, RT.

With the masked aldehyde installed, the anomeric acetyl was removed with dimethyl amine to generate 13. Treatment of 13 with phosphoramidite followed by hydrogen peroxide oxidation yielded a mixture of α- and β-anomers of 14. The separated anomers were examined with the same NOE experiment discussed in Section 2.2.2 (p. 47). The β-anomer of 14 was identified by a strong NOE at H1 that was observed upon selective irradiation of H5 (Figure 2.49), while no such NOE at H1 was observed for the α-anomer (not shown).
The final synthetic steps from 14 to 8 were performed consecutively with care to avoid degradation of the unstable aldehyde intermediates. The alkene was first subjected to ozonolysis and dimethyl sulfide reduction to regenerate the aldehyde, and then the benzyl protecting groups were removed by hydrogenolysis. Hydrolysis of the acetate groups at −20 °C generated 15, which was incubated with HldE at room temperature. The adenylyltransferase activity of HldE readily generated 8, further indicating the tolerance of this activity of HldE to different functional groups at C-6.

The final product 8 exists primarily in the hydrated form in aqueous solution, as indicated by the appearance of the H6″ at 5.16 ppm (Figure 2.50). A full spectrum can be found in the Appendix (Figure A.98, p. 177). No signal arising from to an unhydrated aldehyde was observed in the region from 8 to 11 ppm of the 1H NMR spectrum. The appearance of unknown signals in the anomeric region (marked with a question mark in Figure 2.50) seemed to indicate ADP-sugar impurities had been co-purified with 8, or evolved from 8. It is conceivable that the
basic conditions (pH 11) during deacetylation could have catalyzed epimerization at C-5 to generate the L-gulo-epimer (Figure 2.51). Alternatively, the basic conditions could have promoted elimination of acetic acid from the deprotection intermediate generating an alkene, which is known to occur with related sugars. The α,β unsaturated aldehyde could have accepted hydroxyl at C-4 to generate the D-talo-epimeric product (product of the net syn-addition of water) or L-allo-epimer product (net anti-addition of water). Any of these sugars may have been accepted by HldE to generate an ADP-sugar that co-eluted with 8 during purification by anion exchange chromatography. Consistent with this, a minor signal corresponding to the ADP-alkene product was observed in the mass spectrum of the synthetic mixture of 8 (ESIMS 568.1 m/z [M – H]+).

Figure 2.50 Partial 1H NMR spectrum of ADP-β-D-manno-hexodialdose 8 prepared by the synthesis described in Figure 2.48 (300 MHz, D2O). Unknown signals marked with a question mark are divided into four regions, A-D.

The apparent solution to this problem would be to change the order of steps h, i, and j such that deacetylation precedes the unmasking of the aldehyde. Unfortunately, this is not
possible as the alkene must be oxidized before hydrogenation. In addition, deacetylation must follow hydrogenation, as the protected phosphate is likely to be hydrolyzed during the deacetylation step. Thus, with the present protecting groups the sequencing of unmasking and deprotection of 14 must proceed as shown in Figure 2.48.

Figure 2.51 Possible formation of impurities in the final steps of the synthesis of 8. a) 2:3:0.6 MeOH/0.1M triethylammonium bicarbonate/NEt₃, -20 °C, 4 days. b) ATP, HldE, inorganic pyrophosphatase, RT.

### 2.3.2 Bicyclic Hemiacetal Forms of ADP-β-D-manno-hexodialdose 8

An alternate explanation of the unknown signals in the synthetic mixture of 8 (Figure 2.50) is that they arise from bicyclic hemiacetal forms of 8 involving the C-6'' carbonyl, as in Figure 2.52. In the C₁ conformation of unhydrated 8 the C-3'' hydroxyl and the C-6'' carbonyl of are in equatorial positions, held apart such that formation of a cyclic hemiacetal is not
possible. When the pyranose ring is flipped, the $^{1}C_{4}$ form of unhydrated 8 has both the C-3" hydroxyl and the C-6" carbonyl in axial positions, and a furanose ring can form. The new anomeric centre can exist in either an $\alpha$- or $\beta$-configuration, and are in rapid equilibrium with hydrated 8.

Figure 2.52 Formation of bicyclic forms of 8.

Precedent for the formation of bicyclic 8 comes from literature observations of galactose dialdehyde. NMR spectroscopic studies of $^{13}$C-labeled galactose dialdehyde have determined that 99 % of the dialdehyde exists in the bicyclic forms in neutral aqueous solution (Figure 2.53). While the bicyclic form is the dominant form of galactose dialdehyde, it should be noted that UDP-$\alpha$-D-gluco-hexodialdose was found to exist predominantly as the hydrated aldehyde. Presumably the hydrated form of UDP-$\alpha$-D-gluco-hexodialdose is strongly favoured since the substituents at C-2", C-3", and C-4", and C-5" all may occupy equatorial positions in the $^{4}C_{1}$ conformer.
To discern between the possibilities that the signals at ~5.1 and ~5.6 ppm in the $^1$H NMR spectrum of 8 are due to either synthetic impurities or bicyclic hemiacetals of 8, a new synthetic route to 8 was designed. This route avoids basic conditions in the final steps that might lead to the decomposition pathways discussed above (Figure 2.51). If the $^1$H NMR spectrum of 8 derived from such a route possessed identical signals at ~5.1 and ~5.6 ppm (unknown signals A-D in Figure 2.50), this would be consistent with the hypothesis that these signals are due to bicyclic hemiacetals, and concern about them would be eliminated. To this end, a second synthetic route to 8 was designed that employs protecting groups that can be removed by hydrogenolysis. This synthesis (Figure 2.54) starts from the known compound allyl-2,3,4-tri-O-benzyl-α-D-mannopyranoside (All-2,3,4-O-Bn-Man) which was prepared in four steps from mannose. Swern oxidation generated the C-6 aldehyde, and while this compound was found to survive silica gel chromatography and storage as an oil at 4 °C for several weeks, protection of the crude aldehyde prior to purification gave the highest yield after
the two steps. (R,R)-(−)-Hydrobenzoin was chosen to protect the aldehyde as an acetal as it had previously been successfully used to protect ketones with removal by hydrogenolysis.\textsuperscript{119, 120} Formation of 16 was found to proceed quickly with boron trifluoride diethyl etherate as catalyst, despite the observed formation of a 2,2'-diphenyl-acetaldehyde side-product, generated from the known Pinacol rearrangement of hydrobenzoins.\textsuperscript{121} These conditions were found to give better yields than the transacetalization of the dimethyl acetal with (R,R)-(−)-hydrobenzoin catalyzed by pyridinium p-toluenesulfonate.

![Synthesis of 8 via a hydrogenolysis-labile acetal.](image)

Figure 2.54 Synthesis of 8 via a hydrogenolysis-labile acetal. a) allyl alcohol, BF\textsubscript{3}OEt\textsubscript{2}. b) TrCl, pyr. c) BnBr, NaH, DMF. d) AcOH, H\textsubscript{2}O. e) DMSO, oxalyl chloride, NEt\textsubscript{3}, THF, RT f) (R,R)-(−)-hydrobenzoin, BF\textsubscript{3}OEt\textsubscript{2}. g) PdCl\textsubscript{2}, AcOH, AcOK, H\textsubscript{2}O. h) (BnO)\textsubscript{2}PNEt\textsubscript{2}, 1,2,4-triazole. i) H\textsubscript{2}O\textsubscript{2}, THF. j) H\textsubscript{2}, Pd/C, MeOH. k) ATP, HldE, pyrophosphatase.

Anomeric deprotection of 16 gave the reducing sugar, which was phosphorylated using phosphoramidite followed by oxidation to generate 17 (Figure 2.54). The β-anomer of 17 was identified by a strong NOE between H1′′, H3′′ and H5′′ (Figure 2.55).
Figure 2.55 Identification of the $\beta$-anomer of 17 by NOE difference spectroscopy. Bottom: Partial $^1$H NMR spectrum of 17 (400 MHz, CDCl$_3$). Top: NOE difference spectrum irradiating at 5.42 ppm (400 MHz, CDCl$_3$).

Hydrogenolysis of the hydrobenzoin protecting group from 17 proved to be problematic. It was found that palladium hydroxide was required as the catalytic hydrogenating agent, while palladium did not achieve deprotection at one atmosphere of hydrogen. The observation that the hydrobenzoin acetal is resistant to palladium-catalyzed hydrogenation and requires the more activated palladium hydroxide catalyst is in agreement with the literature.\textsuperscript{120} Unfortunately, under these conditions, reduction of the aldehyde to an alcohol also occurs. As the alcohol byproduct is also a substrate for the adenylyltransferase activity of HldE, and ADP-$\beta$-D-mannose 9 behaves similarly to 8 during anion exchange chromatography, 9 was found to contaminate 8. This is a major shortcoming of the synthesis. To avoid this problem, it would be advantageous to protect the aldehyde as a dibenzyl acetal, which presumably could be deprotected with the aldehyde-compatible palladium on carbon catalyst. However, by carefully monitoring the hydrogenation reaction of 17 the undesired reduction could be minimized, and some aldehyde 8 free of alcohol 9 could be isolated.
Figure 2.56 Partial $^1$H NMR spectrum of 8 prepared via the hydrogenolysis-labile acetal (400 MHz, D$_2$O). Unknown signals A and B are present in the same approximate proportion as in the spectrum of 8 prepared via the first synthetic route (Figure 2.50), suggesting these signals arise from bicyclic hemiacetal forms of 8 rather than from synthetic impurities.

The $^1$H NMR spectrum of 8 generated from this second synthesis also has signals A and B at ~5.1 and ~5.6 ppm, indicating that the appearance of these signals are independent of the synthetic route. In addition, the intensity of these signals is identical in the spectra of 8 derived from two different syntheses. These observations favour the notion that these signals are due to different hemiacetal forms of 8, in equilibrium with the hydrated form.

To confirm this, VT NMR was performed with 8. According to Le Châtelier's principle, raising the temperature of an equilibrium mixture shifts the equilibrium condition in the direction of the endothermic reaction. If the signals A and B (Figure 2.50 and Figure 2.56) are in equilibrium with the signals assigned to hydrated 8 the relative intensities of the signals should change with temperature in a reversible fashion. If the signals A and B are due to impurities or arise from the decomposition of 8 then this should not be true. Thus $^1$H NMR
spectra of 8 (prepared via the hydrogenolysis-labile acetal) were measured at 23, 35, 45 and 24 °C (Figure 2.57). While the most striking change is the downfield shift of the signals at elevated temperature, it is the subtle changes in signal intensity that are relevant here. Close inspection the intensity of the signals A and B increase by ~30-60 % at the expense of the H1'' and H6'' signals, when compared to the ribosyl H1' signal. As 8 is known to be an unstable compound, the temperature range for this experiment was limited to 45 °C. At the end of this experiment the spectrum at 24 °C shows that these spectral changes are reversible and therefore not due to decomposition of 8. This experiment indicates that the compounds that give rise to signals A and B are in equilibrium with the hydrated form of 8.

![NMR spectra of 8](image)

Figure 2.57 VT 1H NMR spectra of 8. From top to bottom: partial 1H NMR spectra as the temperature is increased to 45 °C, then cooled back to 24 °C (400 MHz, D2O. Spectra not referenced). Signals in regions A and B are enhanced at higher temperature while H1'' and H6'' signals are diminished at higher temperature.
In the $^{31}$P NMR spectrum of 8, signals corresponding to minor ADP-sugars are reversibly enhanced at elevated temperatures, consistent with the $^1$H NMR spectroscopic observations (Figure 2.58). The signals appear to correspond to those of ADP-sugars due to the apparent coupling constants and chemical shifts. As the temperature is returned to 24 °C the minor signals return to their original intensity, indicating that the change is reversible and not due to decomposition of 8.

![NMR spectra](image)

Figure 2.58 VT $^{31}$P NMR spectra of 8. From top to bottom: As temperature increases the intensity of minor ADP-sugar signals (*) increases reversibly relatively to the $P\alpha$ and $P\beta$ signals of hydrated 8 (162 MHz, D$_2$O).

These observations are consistent with the hypothesis that hydrated 8 exists in equilibrium with bicyclic hemiacetal forms. Assignment of the $^1$H NMR spectral signals was not completed, however, due to the instability and small amount of 8 obtained. The signals of the mannosyl ring system of these bicyclic hemiacetals could be deconvoluted using NMR techniques such as TOCSY. Elevated temperatures could enhance the intensity of these signals, provided the timescale at which mutarotation occurs does not exceed the NMR timescale.
However, the structural elucidation of the bicyclic hemiacetal forms of 8 does not further the understanding of the mechanism of HldD.

2.3.3 Incubation of ADP-β-d-manno-hexodialdose 8 with HldD

Compound 8 was incubated with HldD and the reaction was first monitored by $^1$H NMR spectroscopy. Upon incubation with catalytic amounts of HldD the most striking spectral change is the time-dependent disappearance of the H6" signal of 8 (Figure 2.59). In addition, the H1" doublet disappears at the same rate as the H6" signal. Two new product doublets of equivalent intensity appear slightly upfield of the H1" signals. Complicated spectral changes were observed in other regions of the $^1$H NMR spectrum, and in the $^{31}$P NMR spectrum; however the described spectral changes between $\delta$ 5.0 and 5.5 ppm proved to be the least convoluted. No spectral changes were observed in a control containing 8 alone. These observations are consistent with HldD-catalyzed dismutation of 8; however the identity of the products is ambiguous in this NMR experiment.

![Figure 2.59 Partial $^1$H NMR spectra during dismutation of 4.2 mM 8 catalyzed by 66 μM HldD at room temperature in 20 mM potassium phosphate pH 7.0 (400 MHz, D$_2$O).](image)
The dismutation products are more easily identified by their distinct masses. As indicated in mass spectrum A in Figure 2.60 aldehyde 8 is observed as the methanol hemiacetal adduct. This is expected since the spectral analysis was performed in methanol. After the HldD catalyzed reaction, 8 was not observed (mass spectrum B, Figure 2.60). Two new signals corresponding to the alcohol 9 and the acid 10 were observed, providing the first strong evidence that the HldD catalyzed reaction of 8 is a dismutation. In accordance with this, the two signals are approximately equal in intensity, as would be expected in a redox balanced dismutation (Figure 2.47, p. 55).

![Figure 2.60 ESI mass spectra of: A) ADP-β-D-manno-hexodialdose 8 as [M - H + MeOH]́. B) ADP-β-D-mannose 9 as [M - 2H + Na]́, and ADP-β-D-mannuronate 10 as [M - 3H + 2Na]́ generated by incubation of 8 with HldD at 37 °C, pH 8 for 12 h. All spectra were taken in MeOH, negative detection mode.](image)

To confirm these assignments, the mixture of products was separated by anion exchange chromatography. As compounds 9 and 10 differ in their charge state at neutral pH this proved to be an efficient method to separate milligram quantities. The spectral properties (¹H and ³¹P NMR spectroscopy, and mass spectra) of alcohol 9 proved to be identical a synthetic sample of 9, which was prepared as described in the following section. The identity of purified 10 was established by mass spectroscopy and ¹H and ³¹P NMR spectroscopy. In addition, the presence
of a carboxylate at C-6" was confirmed with an HMBC (heteronuclear multiple bond correlation) NMR spectroscopy experiment. HMBC spectroscopy is a proton detected correlation spectroscopy that detects heteronuclei more than one bond away. It is similar to HMQC (heteronuclear multiple quantum coherence) spectroscopy that detects heteronuclei directly attached to the proton. In the HMBC experiment of 10, a coupling of the H5" signal to the distinctive C-6" carboxylate carbon signal at 170 ppm was observed, confirming the presence of a carboxylate at C-6" of 10.

### 2.3.4 Synthesis of ADP-β-D-mannose 9

To confirm the identity of the reduction product formed from the apparent dismutation of 8, the anticipated product ADP-β-D-mannose 9 was synthesized as described in Figure 2.61 below. This synthesis bears strong resemblance to the synthesis of 1 and the synthesis of 8. The identity of the β-anomer of 18 has already been established, as 18 is a known compound.\textsuperscript{111} Deprotection of 18 generated 19, which was accepted by the adenylyltransferase activity of HldE to generate alcohol 9.

![Synthesis of ADP-β-D-mannose 9](diagram.png)

Figure 2.61 Synthesis of ADP-β-D-mannose 9. a) Ac\textsubscript{2}O, pyr. b) HNMe\textsubscript{2}, MeCN. c) Dibenzyl N,N-diisopropylphosphoramidite, 1,2,4-triazole, CH\textsubscript{2}Cl\textsubscript{2}. d) 30 % H\textsubscript{2}O\textsubscript{2}, THF, −78 °C. e) H\textsubscript{2}, Pd/C, MeOH, NEt\textsubscript{3}. f) 2:3:0.6 MeOH/0.1M triethylammonium bicarbonate/NEt\textsubscript{3}, −20 °C, 4 days. g) ATP, HldE, inorganic pyrophosphatase, RT.
2.3.5 Observation of HldD-bound NADPH during dismutation

While the observation of dismutation of aldehyde 8 provides evidence that HldD catalyzes redox chemistry at C-6" of ADP-mannosyl sugars, the involvement of the tightly-bound cofactor has not been verified. To confirm its involvement, the redox state of the cofactor was directly monitored by UV spectroscopy. NADPH has a unique 340 nm absorbance band that is well resolved from the 280 nm protein absorbance band, while the absorbance band of NADP$^+$ is not distinct from that of protein. Thus only the reduced form of the enzyme-bound cofactor can be observed directly by UV spectroscopy to confirm the involvement of the cofactor during dismutation.

It has previously been observed that recombinant HldD contains a mixture of reduced and oxidized forms of cofactor upon isolation from *E. coli*. The distinctive NADPH absorption band has been reported at 350 nm, slightly red-shifted from free NADPH due to the fact that it is protein-bound. The UV spectrum of our sample of HldD taken in the absence of substrate (Figure 2.62) also shows a shoulder near 340 nm, indicating that a small portion of HldD contains NADPH. The lack of a distinct NADPH band in this trace is likely due to tailing of the strong 280 nm absorbance band. Immediately after addition of 8 a 354 nm band grows in, indicating that the amount of tightly bound NADPH has been greatly enhanced.
Figure 2.62 Partial UV spectra of HldD-bound NADPH. Bottom trace is the spectrum of HldD alone. Top trace is taken ~30 s after addition of 8. Spectra were collected in 10 mM potassium phosphate buffer, pH 8.0, at 22 °C with [HldD] = 78 μM and initial [8] = 331 μM.

A time-resolved monitoring of the absorbance due to tightly bound NADPH is shown in Figure 2.63. After the initial spike in NADPH immediately following the addition of 8, the concentration of NADPH can be observed to diminish slowly back to the original concentration. This time-scale roughly corresponds to the time scale on which dismutation is observed to occur when monitored by $^1$H NMR spectroscopy. After the decay is complete, the spectrum from 300 nm to 500 nm is identical to that taken before the addition of 8. A difference spectrum of the spectrum taken immediately after addition of 8 minus the spectrum taken after 120 minutes (Figure 2.63, Inset) shows that the greatest spectral change occurs at 354 nm.
Addition of sodium borohydride to HldD results in a similar absorption band at 354 nm, in addition to a 425 nm shoulder (not shown). This is similar to what is observed with the 4,6-dehydratase RmlB, a related SDR enzyme which also tightly binds a nicotinamide cofactor. In that case the 355 nm band is attributed to tightly-bound NADH, and the 425 nm shoulder is attributed to the products of addition of the hydride to the 2- and 6-positions on the nicotinamide ring. This reactivity of sodium borohydride at the 2-, 4-, and 6-positions of the nicotinamide ring is in accordance to the reactivity of sodium borohydride with NAD$^+$ in aqueous solution.

The intensity of the 354 nm absorption band generated upon addition of sodium borohydride to HldD is similar to that observed immediately following addition of 8 to HldD (Figure 2.63). This indicates that nearly all the cofactor is initially driven to the reduced state. Therefore, the rate of oxidation of 8 exceeds the rate of reduction of 8, which is consistent with
the observation that the majority of 8 is in the hydrated form. Oxidation of 8 must necessarily occur via the hydrated form, while reduction must necessarily occur via the unhydrated form. Given that unhydrated 8 is not observed in the $^1$H NMR spectrum of 8, these relative rates more likely reflect the relative abundance of the two species, and not the rate constants of the oxidation and reduction steps.

![Diagram of dismutation](image)

Figure 2.64 Dismutation of 8. Oxidation from the abundant hydrated form of 8 occurs more quickly than reduction of the relatively scarce unhydrated form of 8.

### 2.4 Conclusions

The observation of dismutase activity of HldD with compound 8 is the first direct evidence indicating that HldD operates by a direct C-6" redox mechanism (Figure 2.65). All previous studies provided indirect evidence that was used to rule out other reasonable mechanisms. Those studies included the observation that solvent derived $^2$H and $^{18}$O atoms are not incorporated into substrate during catalysis, that a C-4" deoxy substrate and a C-7" deoxy substrate are proficient substrates for epimerization, and that the C-6" fluorinated analog 1 is not a substrate for HldD. Dismutation of 8 into oxidized and reduced products can only occur by direct C-6" redox chemistry, providing positive evidence that HldD catalyzes oxidation and reduction at C-6". This dismutation of an aldehyde is similar to the Cannizzarro reaction, in which benzaldehyde dismutates into benzyl alcohol and benzoic acid in aqueous alkaline...
conditions. The base-catalyzed dismutation differs fundamentally in that the two aldehyde molecules react via a direct intermolecular reaction. In the enzyme-catalyzed dismutation however, NADP\(^+\) mediates the hydride transfer from one aldehyde to another, as demonstrated by the observation of transient NADPH. This observation of transient NADPH is the first direct evidence that the tightly bound cofactor participates in HldD catalysis.

Figure 2.65 Established direct redox mechanism of HldD.

Since the work described in this chapter was completed, additional evidence supporting the direct redox mechanism has been uncovered by former lab members Dr. Jay Read and Dr. Alain Mayer. Dr. Read found that a C-6\(^{''}\) deuterated substrate (ADP-D,D-Hep containing a deuterium at C-6\(^{''}\)) retains its deuterium label at C-6\(^{''}\) during epimerization (giving ADP-L,D-Hep containing a deuterium at C-6\(^{''}\)).\(^{124}\) This is consistent with a cofactor-mediated delivery of the C-6\(^{''}\) hydride (or deuteride) from one face of the ketone intermediate to the other. Dr. Mayer observed trapping of the ketone intermediate.\(^{125}\) The interconverting C-6\(^{''}\) deuterated substrates were treated with phenylhydrazine and the corresponding phenylhydrazone was observed by mass spectrometry. The absence of \(^{2}\)H in the mass of the phenylhydrazone generated from ADP-[6-\(^{2}\)H]-Hep indicates the trapped intermediate had been oxidized at C-6\(^{''}\).

Several lines of evidence now indicate that HldD operates by the direct redox mechanism. With the chemical mechanism thus established the focus of the project then moved to address questions surrounding the enzyme itself. These questions include, ‘How is the tightly
bound cofactor able to access either face of the ketone carbonyl?' and, 'What are the catalytic residues involved?' These questions are addressed in Chapter Three.

2.5 Experimental

2.5.1 Materials and General Methods

All chemicals were purchased from Sigma-Aldrich and used without further refinement unless otherwise noted. Dry solvents were distilled fresh, using CaH₂ (CH₂Cl₂, pyridine, DMSO) or Na/benzophenone (THF) as drying agent. Inorganic pyrophosphatase (from yeast) was purchased from Roche Diagnostics Corporation. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard.¹²₆ ¹H-NMR spectra were obtained on a Bruker AV300 or AV400 spectrometer at a field strength of 300 or 400 MHz, respectively. Proton-decoupled ³¹P NMR spectra were recorded on these spectrometers at 121.5 MHz or 162 MHz, respectively. Proton-decoupled ¹⁹F NMR spectra were obtained on the Bruker AV300 spectrometer at 282.3 MHz. Mass spectrometry was performed by electrospray ionization (ESIMS) using a Waters Micromass LCT mass spectrometer, and elemental analyses were performed on neutral compounds by Minaz Lahka at the Mass Spectrometry/Microanalysis Laboratory at UBC.

2.5.2 Source of hldD and Cloning of hldE

The hldD gene (formerly rfaD or waaD, GenBank accession number P67910)¹²⁷ had previously been cloned into a pET-30 Xa/LIC vector (Novagen) from Escherichia coli K-12 W3110 genomic DNA template by Dr. Jay Read. The hldE gene (formerly rfaE, GenBank accession number P76658)¹²⁷ was amplified by PCR using Escherichia coli K-12 W3110 genomic DNA as a template. Oligonucleotide primers, synthesized by the NAPS Unit at UBC,
included overhangs for ligation-independent cloning: 5'-GGTATTGAGGTCCATGAAAGTAACGCTGCCAGAG-3' (forward sequence, hldE) and 5'-AGAGGAGAGTTAGAGCCATCTGTGAAACCGCTTTCC-3' (reverse sequence, hldE). A general procedure is as follows: 5.0 µL of 10X PCR buffer (Invitrogen), 1.0 µL of 10 mM dNTP mix, 1.5 µL of 50 mM MgCl₂, ~0.1 µL of E. coli K-12 W3110 cell pellet, 25 pmol of each primer, 0.25 µL of 5 U/µL Taq polymerase, and distilled H₂O to a total volume of 50 µL was added to a 200 µL PCR tube. DNA was amplified using an iCycler Thermal Cycler (Bio-Rad) according to the following cycles: one cycle of 3 min at 94 °C; thirty cycles of 60 s at 94 °C, 60 s at 55 °C, and 90 s at 72 °C; and followed by cooling to 4 °C. The PCR product was cloned into the pET-30 Xa/ LIC vector (Novagen) using the ligation-independent cloning method according to the manufacturer's directions. The resulting plasmid, which encodes for the protein fused to an N-terminal 43-residue peptide containing a hexahistidine tag, was transformed into NovaBlue Gigasingles chemically competent E. coli cells (Novagen). The presence of the gene was confirmed by colony PCR and DNA sequencing.

2.5.3 Overexpression and Purification of Histidine-Tagged HldD and HldE

Overexpression of HldD and HldE was performed as described in the following generic procedure. The recombinants were transformed into expression host cells, JM109(DE3) chemically competent E. coli, which were incubated overnight at 37 °C with shaking at 225 rpm in 10 mL Luria-Bertani (LB) medium containing 30 µg mL⁻¹ kanamycin. The overnight cultures were poured into 500 mL LB medium containing 30 µg mL⁻¹ kanamycin and grown at 37 °C with shaking at 225 rpm until an OD₆₀₀ of 0.6-0.9 was reached. Cells were induced for overexpression by addition of 120 mg L⁻¹ (0.5 mM) IPTG, and the cultures were allowed to continue growth until an OD₆₀₀ of 1.6-1.8 was reached (~6 h). Cells were harvested at 4 000
rpm for 30 min, and resuspended in lysis buffer. In the case of HldD, TEM lysis buffer was used (10 mM Tris-HCl, 2.5 mM EDTA, pH 8.0, 5 mM β-mercaptoethanol) with 1 μg mL⁻¹ pepstatin A, and 1 μg mL⁻¹ aprotinin added. The buffer used for HldE was 20 mM triethanolamine-HCl (pH 8.0) with 1 mM DTT, 1 μg mL⁻¹ pepstatin A, and 1 μg mL⁻¹ aprotinin added. Cells were lysed at 20 000 psi in an ice-cooled French pressure cell. The cell lysate was clarified by centrifugation at 6 000 rpm for 40 min and filtered through a 0.4 μM membrane prior to affinity chromatography.

A 10 mL column containing Chelating Sepharose Fast Flow resin (Pharmacia Biotech) was charged with 2 column volumes (CV) of 100 mM NiSO₄, followed by washing with 2 CV of distilled H₂O and 3 CV of running buffer (lysis buffer minus aprotinin and pepstatin plus 500 mM NaCl) containing 5 mM imidazole. The filtered cell lysate was loaded at 2 mL min⁻¹, and start buffer (~8 CV) was passed through the column at 3 mL min⁻¹ until no more flow-through protein eluted, as determined by UV observation at 280 nm. A wash with running buffer containing 125 mM imidazole was used to remove non-specifically bound proteins (~4 CV used). Histidine-tagged protein was finally eluted with 3-4 CV of running buffer containing 500 mM imidazole. Fractions containing the enzyme were pooled and dialyzed overnight against a 1:100 volume of dialysis buffer. In the case of HldD the buffer was 10 mM Tris-HCl, pH 8.0, containing 2.5 mM EDTA, 5 mM β-mercaptoethanol, and 10 % glycerol. In the case of HldE the buffer was 20 mM triethanolamine-HCl, pH 8.0, containing 1 mM DTT and 10 % glycerol. Enzymes were concentrated by ultrafiltration (Amicon Ultra-4, 10 000 MWCO) to 8-20 mg mL⁻¹, and 25 to 500 μL aliquots were flash-frozen in liq. N₂ and stored at −80 °C. Enzymes could be stored at −80 °C for at least 12 months without significant loss in activity.
2.5.4 Standard Procedures

2.5.4.1 Anomeric Deacetylation with Dimethylamine

The fully acetylated sugar (1 mmol) is dissolved in acetonitrile (~5 mL per 1 mmol sugar) and dimethyl amine is added (5 mmol, 5 M solution in ethanol, Fluka). The solution is allowed to stir at room temperature and the reaction is followed by tlc. Typically the reaction is complete after 90 min. The solvent is then removed by rotary evaporation, and the product is purified by column chromatography.

2.5.4.2 Phosphitylation with Dibenzy l N,N-Diethylphosphoramidite and Oxidation with Hydrogen Peroxide

This procedure is adapted from the literature. To a solution of 1 mmol anomerically deprotected sugar and 4 mmol (72 mg) 1,2,4-triazole in 5.0 mL dry CH$_2$Cl$_2$ under an argon atmosphere, 2.50 mmol (880 µL) 85 % dibenzyl N,N-diethylphosphoramidite is added. After stirring at room temperature for 2 h, 30 mL diethyl ether is added and the organic solution is washed with saturated sodium bicarbonate solution (3 x 15 mL) and NaCl brine (3 x 10 mL). The organic layer is dried over sodium sulphate and evaporated to an oil. The oil is dissolved in 9 mL THF and cooled to −78 °C, then 1.9 mL 30 % H$_2$O$_2$ is added and the solution was allowed to warm to room temperature over 2 h. Diethyl ether (30 mL) is then added and the organic solution is washed with saturated sodium bicarbonate solution (3 x 15 mL) and NaCl brine (3 x 10 mL). The organic layer is dried over sodium sulphate and evaporated to give an oil which is then purified by column chromatography.
2.5.4.3 Hydrogenolysis of Benzyl Protecting Groups from Phosphate

The sugar is dissolved in MeOH (10 mL per 100 mg sugar) and two equivalents of triethylamine are added. Carefully, 5 % Pd/C is added (50 % by weight). The solution is vigorously stirred under one atmosphere H₂ until judged complete by tlc (usually overnight). The reaction mixture is then filtered through celite and the solvent is removed in vacuo.

2.5.4.4 Deacetylation of β-Mannosyl Phosphates

This procedure has been adapted from the literature. The acetylated β-mannosyl phosphate (0.2 mmol) is dissolved in 3 mL of cold (–20 °C) 3:4:0.3 methanol/0.1 M triethylammonium bicarbonate buffer/triethylamine (pH 11) and allowed to sit at –20 °C for 4 days. The solution is then diluted three-fold with water, frozen, and lyophilized to dryness.

2.5.4.5 Formation of ADP-β-D-mannosyl Sugar Nucleotides from β-Mannosyl Phosphates with HldE Adenylyltransferase Activity

The deprotected β-mannosyl phosphate (0.2 mmol) is dissolved in 6 mL of a solution containing 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM MgCl₂, ATP (0.2 mmol), 400 μg HldE and 20 μg inorganic pyrophosphatase and is allowed to sit at room temperature. Progress of this reaction may be monitored by ³¹P NMR spectroscopy, however usually the reaction is complete after overnight incubation. Separation by anion exchange column chromatography (DE-52 resin, linear gradient from 0.1 M to 0.5 M of triethylammonium bicarbonate buffer, pH 7.5) affords the fractions containing the ADP-sugar which are flash frozen and lyophilized with care to ensure no melting of partially lyophilized samples occurred. Residual triethylamine can be removed by dissolving the lyophilized solid in water immediately followed by freezing and lyophilization.
2.5.5 Specific Synthetic Procedures

2.5.5.1 (2'S,3'S)-Methyl-6-deoxy-3,4-O-(2',3'-dimethoxybutane-2',3'-diyl)-6-fluoro-α-D-mannopyranoside 3

(2'S,3'S)-Methyl 3,4-O-(2',3'-dimethoxybutane-2',3'-diyl)-α-D-mannopyranoside 2 was prepared as described in the literature.128 Compound 2 (400 mg, 1.30 mmol) was dissolved in 6 mL dry CH$_2$Cl$_2$ in a flame-dried flask and chilled under argon to −40 °C in a dry ice/acetonitrile bath. DAST (0.19 mL, 1.4 mmol) was added slowly via syringe and the solution was stirred at −40 °C for one hour, and then allowed to warm to room temperature over one hour. The solution was then cooled to −10 °C and 0.5 mL of methanol was added. The solvent was then removed by rotary evaporation and the crude mixture separate by silica gel chromatography. Silica gel column chromatography (100 % CH$_2$Cl$_2$ to 1:1 CH$_2$Cl$_2$/ethyl acetate) yielded 85 mg (2'S,3'S)-methyl-6-deoxy-3,4-O-(2',3'-dimethoxybutane-2',3'-diyl)-6-fluoro-α-D-mannopyranoside 3 (R$_f$ = 0.6 in 1:1 CH$_2$Cl$_2$/ethyl acetate, 0.27 mmol, 21 % yield). $^1$H NMR (CDCl$_3$): δ 4.77 (d, 1H, $J_{1,2} = 1.1$ Hz, H1), 4.65 (ddd, 1H, $J_{5,6'} = 3.9$ Hz, $J_{6',6''} = 10.0$ Hz, $J_{6'',F} = 47.4$ Hz, H6'), 4.60 (ddd, 1H, $J_{5,6''} = 1.9$ Hz, $J_{6',6''} = 10.4$ Hz, $J_{6'',F} = 47.8$ Hz, H6''), 4.10 (dd, 1H, $J_{3,4} = 10.0$ Hz, $J_{4,5} = 10$ Hz, H4), 4.01 (dd, 1H, $J_{2,3} = 3.1$ Hz, $J_{3,4} = 10.0$ Hz, H3), 3.93 (dd, 1H, $J_{1,2} = 1.5$ Hz, $J_{2,3} = 3.1$ Hz, H2), 3.86 (ddd, $J_{4,5} = 10$ Hz, $J_{5,6'} = 3.9$ Hz, $J_{5,6''} = 1.9$ Hz, $J_{5,F} = 25.8$ Hz, H5), 3.39, 3.28, 3.24 (3 x s, 3 x 3H, 3 x OMe), 1.33, 1.29 (2 x s, 2 x 3H, 2 x Me); $^{19}$F NMR (CDCl$_3$): δ −172.8 (s); ESIMS: $m/z$ 333.3 [M + Na]$^+$; Anal. calcd. for C$_{13}$H$_{23}$FO$_7$: C, 50.32 %, H 7.47 %, Found: C, 50.43 %, H 7.48 %.
To a solution of 543 mg compound 3 (1.75 mmol) in 18 mL chloroform, 2 mL TFA was added and the solution was stirred at room temperature for two hours. The solvent was then removed by rotary evaporation to give a yellow oil with a strong butter-like odor. The crude oil was dissolved in 6 mL acetic anhydride and 0.1 mL conc. sulphuric acid was added. After stirring for two hours 10 mL CHCl₃ was added, then 10 mL H₂O was slowly added with vigorous stirring. In a large beaker saturated sodium bicarbonate was then slowly added with vigorous stirring until the evolution of bubbles subsided. Extraction with 3 x 30 mL ethyl acetate was followed by washing of the pooled organic layers with 2 x 20 mL saturated sodium bicarbonate solution. The organic layer was dried over anhydrous MgSO₄ and the solvent was removed in vacuo to give 455 mg crude residue. Silica gel chromatography (eluted with 100 % CH₂Cl₂ to 19:1 CH₂Cl₂/ethyl acetate) gave 308 mg (0.88 mmol) 1,2,3,4-tetra-O-acetyl-6-deoxy-6-fluoro-D-mannopyranose 4 as a mixture of anomers in 50 % yield (Rf 0.5 in 19:1 CH₂Cl₂/ethyl acetate). The identity of 4 was confirmed by mass spectrometry (ESIMS: m/z 373.2 [M + Na]⁺) and the matching of its ¹H and ¹⁹F NMR spectra to that reported in the literature. Anomeric deacetylation of 643 mg (1.84 mmol) of 4 using the standard dimethylamine procedure followed by column chromatography (gradient of 100 % CH₂Cl₂ to 4:1 CH₂Cl₂/ethyl acetate) gave 495 mg (1.61 mmol, 87 % yield) of 2,3,4-tri-O-acetyl-6-deoxy-6-fluoro-D-mannopyranose 5 (Rf = 0.3 in 4:1 CH₂Cl₂/ethyl acetate). The identity 5 was confirmed by mass spectrometry (ESIMS: m/z 331.4 [M + Na]⁺). Installation of the anomeric phosphate was accomplished using the standard procedure using dibenzyl N,N-diethylphosphoramide. This generated a mixture of α- and β-anomers, which were separated by column chromatography (gradient of 100 % CH₂Cl₂ to 4:1 CH₂Cl₂/ethyl acetate, α-anomer
R\text{f} = 0.4, \beta\text{-anomer R}\text{f} = 0.2 \text{ in 17:3 CH}_2\text{Cl}_2/\text{ethyl acetate}). The anomers were distinguishable by NOE (Figure 2.43, p. 50). From 400 mg 5 (1.30 mmol), 130 mg (0.229 mmol) of purified dibenzyl 2,3,4-tri-O-acetyl-6-deoxy-6-fluoro-\beta-D-mannopyranose phosphate 6 was obtained (18 % yield). $^1$H NMR (CDCl$_3$): \(\delta 7.32-7.39 \text{ (m, 10H, 2 x Ph), 5.48 (s, 1H, H1), 5.46 (d, 2H, J_{2,3} = 3.1 \text{ Hz, H2}), 5.27 (dd, 1H, J_{3,4} = 9.8 \text{ Hz, J}_{4,5} = 9.8 \text{ Hz, H4}), 5.12-5.08 \text{ (m, 4H, 2 x CH}_2\text{Ph), 5.06 (dd, 1H, J}_{2,3} = 3.7 \text{ Hz, J}_{3,4} = 8.3 \text{ Hz, H3}), 4.48 (dd, 2H, J_{6,F} = 47.0 \text{ Hz, J}_{5,6} = 3.9 \text{ Hz, H6}), 3.77 (ddt, 1H, J_{4,5} = 9.6 \text{ Hz, J}_{5,6} = 3.9 \text{ Hz, J}_{5,F} = 20.4 \text{ Hz, H5}), 2.15, 2.08, 2.01 (3 x s, 3 x 3H, 3 x COCH}_3);$ $^{19}$F NMR (CDCl$_3$): \(\delta -154.5 \text{ (s)};{^{31}}P \text{ NMR (CDCl}_3): {\delta -1.64 \text{ (s)}}; \text{ESIMS}: m/z 591.2$ $[\text{M + Na}]^+; \text{Anal. calcd. for C}_{26}\text{H}_{30}\text{FO}_{11}\text{P: C, 54.93 \%}, \text{H 5.32 \%}, \text{Found: C, 54.75 \%, H 5.43 \%}.$

### 2.5.5.3 Adenosine Diphospho-6-deoxy-6-fluoro-\beta-D-mannose 1,

**Bis(triethylamine) Salt**

The benzyl protecting groups were removed from 44 mg (77 \(\mu\)moles) of 6 using the standard hydrogenolysis procedure. The removal of the benzyl protecting groups was confirmed by $^1$H NMR of the crude product (in CD$_3$OD), and the crude product was then further deprotected using the standard deacetylation procedure. The crude fully deprotected product was then incubated with HldE as described in the standard procedure. The purified product was quantitated by UV measurement at 259 nm to measure 50 \(\mu\)moles of adenosine diphospho-6-deoxy-6-fluoro-\beta-D-mannose 1 (65 \% yield). $^1$H NMR (D$_2$O): \(\delta 8.49 \text{ (s, 1H, H8), 8.24 (s, 1H, H2), 6.12 (d, 1H, J_{1',2'} = 5.8 \text{ Hz, H1'}), 5.25 (d, 1H, J_{1'',pB} = 8.9 \text{ Hz, H1''}), 4.78-4.70 \text{ (obscured by solvent peak, H2'')}, 4.78 - 4.70 + 4.57 \text{ (dd, partly obscured by solvent peak, J}_{5',6'} = 2.3 \text{ Hz, H6''}), 4.51 \text{ (dd, 1H, J}_{2',3'} = 5.0 \text{ Hz, J}_{3',4'} = 3.5 \text{ Hz, H3''}), 4.37 \text{ (dd, 1H, J}_{4',5'} = 2.5 \text{ Hz, J}_{4',5'} = 2.5 \text{ Hz, H5'}), 4.20 \text{ (dd, 2H, J}_{5',pD} = 5.2 \text{ Hz, J}_{4',5'} = 3.3 \text{ Hz, H5'}), 4.08 (m, 1H, H2'''), 3.71-3.65 (m, 2H, H3''' and H4'''), 3.39-3.56 (m, 1H, H5'''), 3.14 (q, 12H, J = 7.3 Hz,} 83
[HN(CH₂CH₃)₃]⁺, 1.24 (t, 18H, J = 7.3 Hz, [HN(CH₂CH₃)₃]⁺); ⋅¹⁹F NMR (D₂O): δ -157.6 (s); ⋅³¹P NMR (D₂O): δ -10.2 (d, 1P, Jₚα,ₚβ = 20.3 Hz, Pα), -12.3 (d, 1P, Jₚα,ₚβ = 20.3 Hz, Pβ); ESIMS: m/z 612.1 [M - 2H + Na]⁻.

2.5.5.4 1,2,3,4-Tetra-O-acetyl-6,7-dideoxy-β-D-manno-hept-6-enopyranose 12

To a solution of 3.00 g (8.61 mmol) 11¹¹⁵ in 45 mL dry DMSO under argon, 11.0 g (26.0 mmol) of N-cyclohexyl-N'-(2-morpholinoethyl)carbodiimide methyl-p-toluenesulfonate (CMC, Fluka) was added. To this solution 1.1 mL dichloroacetic acid (13.4 mmol) was added via syringe and the resulting solution was stirred in a cool water bath (~10 °C). After 3 h the reaction mixture was poured into 250 mL ice-cold distilled water and extracted with ice-cold ethyl acetate (4 x 100 mL). The pooled organic layers were extracted with ice-cold distilled water (3 x 100 mL), and the pooled aqueous layers were back-extracted with 100 mL ice-cold ethyl acetate. The pooled organic layers were then washed with ice-cold NaCl brine and dried over sodium sulfate. Evaporation of the solvent gave 2.43 g of pale yellow residue. The crude aldehyde product was used immediately without further purification in the following methylenation reaction with the Lombardo reagent. The Lombardo reagent was prepared by cooling to -40 °C a solution of 11.5 g (17.6 mmol) zinc dust in 100 mL dry THF with 4.05 mL (8.0 mmol) dibromomethane and adding drop-wise 4.6 mL (5.7 mmol) neat TiCl₄. The Lombardo reagent was allowed to stir at -40 °C for 2 h, then at 4 °C for two days prior to use. The entire crude aldehyde product was dissolved in 50 mL dry methylene chloride and cooled on ice. The entire Lombardo reagent was added to the aldehyde solution and stirred for 30 minutes at 0 °C. The reaction mixture was then poured into 250 mL saturated sodium bicarbonate solution with 500 mL ethyl acetate and stirred vigorously until evolution of gas...
dissipated (~1 hour). The resulting mixture was filtered through celite, and the two layers separated. The aqueous layer was extracted with ethyl acetate (2 x 100 mL). The combined organic layers were washed with 200 mL water, brine (3 x 100 mL), and dried over sodium sulfate. Evaporation of the solvent gave 1.61 g yellow residue. Silica gel column chromatography (3:1 petroleum ether/ethyl acetate) gave 343 mg 1,2,3,4-tetra-O-acetyl-6,7-dideoxy-β-D-manno-hept-6-enopyranose 12 as a white solid (R_f = 0.15, 0.995 mmol, 11.6 % yield). _1^H NMR (CDCl_3): δ 5.89 (d, 1H, J_{1,2} = 1.2 Hz, H1), 5.80 (ddd, 1H, J_{5,6} = 7.2 Hz, J_{6,7a} = 17.2 Hz, J_{6,7b} = 10.4 Hz, H6), 5.49 (dd, 1H, J_{1,2} = 1.2 Hz, J_{2,3} = 3.2 Hz, H2), 5.38 (d, 1H, J_{6,7a} = 17.2 Hz, H7a), 5.30 (d, 1H, J_{6,7b} = 10.4 Hz, H7b), 5.19 (dd, 1H, J_{3,4} = 10.0 Hz, J_{4,5} = 10.0 Hz, H4), 5.13 (dd, 1H, J_{2,3} = 2.8 Hz, J_{3,4} = 10.0 Hz, H3), 3.94 (dd, 1H, J_{4,5} = 8.2 Hz, J_{5,6} = 8.2 Hz, H5), 2.21, 2.10, 2.01, 2.00 (4s, 12H, 4 x OCH_3); ESIMS m/z 367.2 [M + Na]^+; Anal. Calcd for C_{15}H_{20}O_{9}: C, 52.32 %, H, 5.85 %. Found: C, 52.46 %, H, 6.24 %.

2.5.5.5 2,3,4-Tri-O-acetyl-6,7-dideoxy-D-manno-hept-6-enopyranose 13

Using the standard procedure for anomeric deacetylation described above 421 mg (1.22 mmol) 12 was anomerically deacetylated. Silica gel column chromatography (5:2 petroleum ether/ethyl acetate) yielded 314 mg of 2,3,4-tri-O-acetyl-6,7-dideoxy-D-manno-hept-6-enopyranose 13 (R_f = 0.3, 1.04 mmol, 85.2 % yield). _1^H NMR (CDCl_3)(−7:1 ratio of α- to β-anomers, as determined by integration of H5 signals. Assignment of the α- and β-anomeric signals is based upon selective NOE and COSY experiments. α-Anomer: δ 5.78 (ddd, 1H, J_{5,6} = 7.6 Hz, J_{6,7a} = 17.2 Hz, J_{6,7b} = 10.4 Hz, H6), 5.40 (dd, 1H, J_{2,3} = 3.4 Hz, J_{3,4} = 10.2 Hz, H3), 5.33 (d, 1H, J_{6,7a} = 17.2, H7a), 5.26 (d, 1H, J_{2,3} = 3.2 Hz, H2), 5.25 (d, 1H, J_{6,7b} = 10.4 Hz, H7b), 5.19 (s, 1H, H1), 5.15 (dd, 1H, J_{3,4} = 10.2 Hz, J_{4,5} = 10.2 Hz, H4), 4.39 (dd, 1H, J_{4,5} = 9.6 Hz, J_{5,6} = 8.0 Hz, H5), 2.13, 1.98, 1.97 (3s, 9H, 3 x OAc); β-anomer: δ 5.83-5.73 (H6, signals
overlapped by H6 of the α-anomer), 5.42-5.05 (H2, H3, H4, H7a, H7b, signals overlapped by
those of the α-anomer), 4.97 (s, 1H, H1), 3.85 (dd, \( J_{4,5} = 8.0 \text{ Hz}, J_{5,6} = 8.0 \text{ Hz}, \) H5), 2.19 (s, 3H, OAc), 1.98, 1.97 (2 x OAc, signals overlapped by those of the α-anomer); ESIMS \( m/z \) 325.0 [M + Na]^+; Anal. Calcd for C_{15}H_{18}O_{8}: C, 51.65 %, H, 6.00 %. Found: C, 52.26 %, H, 5.87 %.

2.5.5.6 Dibenzyl 2,3,4-Tri-O-acetyl-6,7-dideoxy-β-D-manno-hept-6-enopyranosyl Phosphate 14

The standard phosphitylation and oxidation procedures described above were scaled
accordingly for 291 mg (0.964 mmol) 13. The crude product was purified by column
chromatography (1:1 petroleum ether/diethyl ether to 100 % diethyl ether, silica gel) which
gave 133 mg of dibenzyl 2,3,4-tri-O-acetyl-6,7-dideoxy-β-D-manno-hept-6-enopyranosyl
phosphate 14 as a pale yellow oil (Rf = 0.4 in 100 % diethyl ether, 0.244 mmol, 25.3 % yield).
\( ^1H \) NMR (CDCl₃): \( \delta \) 7.33 (m, 10H, Ph), 5.80 (ddd, 1H, \( J_{5,6} = 7.2 \text{ Hz}, J_{6,7a} = 17.2 \text{ Hz}, J_{6,7b} = 10.4 \text{ Hz}, \) H6), 5.48-5.45 (m, 2H, H1 and H2), 5.35 (d, 1H, \( J_{6,7a} = 16.6 \text{ Hz}, \) H7a), 5.28 (d, 1H, \( J_{6,7b} = 10.4, \) H7b), 5.16 (dd, 1H, \( J_{3,4} = 9.8 \text{ Hz}, J_{4,5} = 9.8 \text{ Hz}, \) H4), 5.08-5.02 (m, 5H, PhCH₂ and H3), 3.92 (dd, 1H, \( J_{4,5} = 9.5 \text{ Hz}, J_{5,6} = 7.4 \text{ Hz}, \) H5), 2.15, 2.01, 2.00 (3s, 9H, OAc); \( ^{31}P \) NMR (CDCl₃): \( \delta \) −1.65 (s); ESIMS \( m/z \) 585.2 [M + Na]^+; Anal. Calcd for C_{27}H_{31}O_{11}P: C, 57.65 %, H, 5.55 %. Found: C, 58.04 %, H, 5.76 %.

2.5.5.7 Adenosine Diphospho-β-D-manno-hexodialdose 8,
Bis(triethylamine) Salt (From 14)

A solution of 19.7 mg 14 (0.036 mmol) in 10 mL methylene chloride was cooled to −78
°C in a dry ice/acetone bath, then ozone was bubbled through until a faint blue colour persisted
in the solution (~5 min). Dimethyl sulfide (1 mL) was then added, and the solution was allowed
to warm to room temperature. Upon evaporation of the solvent the subsequent hydrogenolysis
was performed without delay. The crude aldehyde was dissolved in 10 mL 1:1 methanol/ethyl acetate and 35 mg 5 % Pd/C was added. The solution was vigorously stirred under one atmosphere $H_2$ for one hour. The reaction mixture was then filtered through celite and the solvent was evaporated. The crude product was immediately subjected to deacetylation using the standard procedure. The resulting residue was then incubated with HldE and purified by anion exchange chromatography using the standard procedure. This afforded 0.021 mmol adenosine diphospho-β-D-manno-hexodialdose 8 (as determined by UV measurement at 259 nm), 58 % yield. $^1$H NMR (D$_2$O): $\delta$ 8.49 (s, 1H, H8), 8.25 (s, 1H, H2), 6.12 (d, 1H, $J_{1',2'} = 6.0$ Hz, H1'), 5.25 (d, 1H, $J_{1',pH} = 8.8$ Hz, H1''), 5.16 (d, 1H, $J_{5',6'} = 2.0$ Hz, H6''), 4.70-4.80 (obscured by solvent peak, H2''), 4.51 (dd, 1H, $J_{2',3'} = 5.1$ Hz, $J_{3',4'} = 3.5$ Hz, H3''), 4.38 (dd, 1H, $J_{3',4'} = 6.0$ Hz, H1''), 5.25 (d, 1H, $J_{1',pH} = 8.8$ Hz, H1''), 5.16 (d, 1H, $J_{5',6'} = 2.0$ Hz, H6''), 4.70-4.80 (obscured by solvent peak, H2''), 4.51 (dd, 1H, $J_{2',3'} = 5.1$ Hz, $J_{3',4'} = 3.5$ Hz, H3''), 4.38 (dd, 1H, $J_{3',4'} = 6.0$ Hz, H1''), 5.25 (d, 1H, $J_{1',pH} = 8.8$ Hz, H1''), 5.16 (d, 1H, $J_{5',6'} = 2.0$ Hz, H6''), 4.70-4.80 (obscured by solvent peak, H2''), 4.51 (dd, 1H, $J_{2',3'} = 5.1$ Hz, $J_{3',4'} = 3.5$ Hz, H3''), 4.38 (dd, 1H, $J_{3',4'} = 6.0$ Hz, H1''), 5.25 (d, 1H, $J_{1',pH} = 8.8$ Hz, H1''), 5.16 (d, 1H, $J_{5',6'} = 2.0$ Hz, H6''), 4.70-4.80 (obscured by solvent peak, H2''), 4.51 (dd, 1H, $J_{2',3'} = 5.1$ Hz, $J_{3',4'} = 3.5$ Hz, H3''), 4.38 (dd, 1H, $J_{3',4'} = 6.0$ Hz, H1''), 5.25 (d, 1H, $J_{1',pH} = 8.8$ Hz, H1''), 5.16 (d, 1H, $J_{5',6'} = 2.0$ Hz, H6''), 4.70-4.80 (obscured by solvent peak, H2''), 4.51 (dd, 1H, $J_{2',3'} = 5.1$ Hz, $J_{3',4'} = 3.5$ Hz, H3''), 4.38 (dd, 1H, $J_{3',4'} = 6.0$ Hz, H1''), 5.25 (d, 1H, $J_{1',pH} = 8.8$ Hz, H1''), 5.16 (d, 1H, $J_{5',6'} = 2.0$ Hz, H6''),

### 2.5.5.8 1-Allyl-2,3,4-tri-O-benzyl-6,6-(R,R)-hydrobenzoin-α-D-manno-hexodialdose

A solution of 1.30 mL (18.3 mmol) distilled DMSO in 10 mL distilled THF was cooled to $-40$ °C under argon atmosphere. Dropwise 0.96 mL oxalyl chloride (11 mmol) was added and the solution was stirred for 30 min at $-40$ °C. A solution of 1.80 g (3.67 mmol) 1-allyl-2,3,4-tri-O-benzyl-α-D-mannose$^{118}$ in 15 mL dry THF was cannulated into the first solution and the reaction solution was stirred for 30 min at $-40$ °C. At this time 2.55 mL triethylamine (18.3
mmol) in 10 mL dry THF was cannulated to the reaction vessel and the reaction solution was allowed to slowly warm to room temperature over 45 min, at which time the reaction was worked up as follows. The reaction mixture was added to 50 mL CHCl₃ and washed with 3 x 40 mL H₂O. The combined aqueous layers were extracted with 3 x 30 mL CHCl₃ and the combined organic layers were washed with 30 mL NaCl brine. The organic layer was dried over MgSO₄ and the solvent was removed in vacuo. This gave 1.93 g of crude aldehyde product (< 3.67 mmol), which was combined with 1.18 g (R,R)-(+)-hydrobenzoin (5.51 mmol) and 15 mL distilled CH₂Cl₂ added. Upon addition of 698 μL boron trifluoride diethyl etherate (5.51 mmol) a yellow-orange solution formed instantly. After 10 min 5 mL triethylamine was added and the solvent was removed in vacuo. Silica gel chromatography (9:1 petroleum ether/ethyl acetate) afforded 1.39 g of 1-Allyl-2,3,4-tri-O-benzyl-6,6-(R,R)-hydrobenzoin-α-D-manno-hexodialdose 16 (Rₜ = 0.3, 2.03 mmol, 55 % yield). ¹H NMR (400 MHz, CDCl₃): δ 7.43-7.25 (m, 25H, Ph), 6.00-5.90 (m, 1H, All), 5.93 (d, 1H, J₅,₆ = 1.1 Hz, H6), 5.32 (dd, 1H, J = 1.5, 17.2 Hz, All), 5.24 (dd, 1H, J = 1.2, 10.4 Hz, All), 5.08 (d, 1H, J₁,₂ = 1.3 Hz, H1), 5.04-4.70 (m, 8H, CH₂Ph and (CHPh)₂), 4.36-4.31 (m, 1H, All), 4.30 (dd, 1H, J₃,₄ = 9.6 Hz, J₄,₅ = 9.6 Hz, H4), 4.13-4.07 (m, 1H, All), 4.07 (dd, 1H, J₂,₃ = 3.2 Hz, J₃,₄ = 9.2 Hz, H3), 3.99 (dd, 1H, J₄,₅ = 9.9 Hz, J₅,₆ = 0.9 Hz, H5), 3.91 (dd, 1H, J₁,₂ = 1.9 Hz, J₂,₃ = 2.8 Hz, H2); ESIMS m/z 707.3 [M + Na]+. Anal. calcd. for C₄₄H₄₄O₇: C, 77.17 %, H, 6.48 %. Found: C, 77.18 %, H, 6.53 %.

2.5.5.9 Dibenzyl 2,3,4-Tri-O-benzyl-6,6-(R,R)-hydrobenzoin-β-D-manno-hexodialdose Phosphate 17

To a solution of 1.22 g 16 (1.78 mmol) in 7 mL acetic acid, 8 drops of H₂O, 453 mg PdCl₂ (5.52 mmol), and 453 mg sodium acetate (2.55 mmol) were added to generate a brown mixture which was stirred for 24 h (RT). The solvent was removed in vacuo and the crude product was resuspended in 100 mL ethyl acetate, filtered, and washed with 3 x 50 mL aqueous
saturated sodium bicarbonate, then washed with 50 mL sodium chloride brine. The organic layer was dried over MgSO₄ and the solvent was removed in vacuo. Silica gel column chromatography (6:1→3:1 petroleum ether/ethyl acetate) afforded 962 mg (1.49 mmol, 84 % yield) 2,3,4-tri-O-benzyl-6,6-(R,R)-hydrobenzoin-ß-manno-hexodialdose (Rf = 0.2 in 3:1 petroleum ether/ethyl acetate), as suggested by ¹H NMR spectroscopy. The sugar (962 mg, 1.49 mmol) was then subjected to the standard phosphitylation with dibenzyl N,N-diethylphosphoramidite and oxidation with hydrogen peroxide procedure. Silica gel column chromatography (2:1→1:2 petroleum ether/diethyl ether) afforded 231 mg dibenzyl 2,3,4-tri-O-benzyl-6,6-(R,R)-hydrobenzoin-ß-manno-hexodialdose phosphate 17 (Rf = 0.6 in diethyl ether, 0.255 mmol, 17 % yield). ¹H NMR (400 MHz, CDCl₃): δ 7.37-7.05 (m, 35H, Ph), 5.90 (d, 1H, J₅,₆ = 3.0 Hz, H₆), 5.43 (dd, 1H, J₁,₂ = 1.1 Hz, J₁,p = 7.5 Hz, H₁), 5.11-4.56 (m, 12H, CH₂Ph and (CH₂Ph)₂), 4.21 (dd, 1H, J₃,₄ = 8.3 Hz, J₄,₅ = 8.3 Hz, H₄), 3.89 (dd, 1H, J₁,₂ = 1.3 Hz, J₂,₃ = 2.7 Hz, H₂), 3.76 (dd, 1H, J₄,₅ = 8.3 Hz, J₅,₆ = 3.0 Hz, H₅), 3.64 (dd, 1H, J₂,₃ = 3.0 Hz, J₃,₄ = 8.4 Hz, H₃); ³¹P NMR (CDCl₃): δ -2.89 (s); ESIMS: m/z 927.6 [M + Na]⁺; Anal. calcd. for C₁₃H₂₃FO₇: C, 73.00 %, H 5.90 %, Found: C, 72.60 %, H 5.96 %.

### 2.5.5.10 Adenosine Diphospho-β-manno-hexodialdose 8, Bis(triethylamine) Salt (From 17)

To a solution of 20 mg 17 (22 nmol) and 4.6 μL triethylamine (33 nmol) in 10 mL 1:1 methanol/ethyl acetate, 50 mg 20 % Pd(OH)₂/C was added and allowed to stir under one atmosphere of H₂ for 16 h. The reaction mixture was then filtered through celite and the solvent was removed in vacuo. The crude product was then subjected to the adenylyltransferase activity of HldE following the standard procedure. Following anion exchange chromatography 10 nmol
of adenosine diphospho-β-D-manno-hexodialdose 8 (as determined by UV spectroscopy) was isolated (45 % yield).

2.5.5.11  Adenosine Diphospho-β-D-mannose 9, Bis(triethylamine) Salt

Peracetylation of D-mannose\textsuperscript{130} followed by anomeric deacetylation using dimethyl amine (standard procedure) gave 2,3,4,6-tetra-O-acetyl-D-mannopyranose (2,3,4,6-O-Ac-Man) as determined by comparison of $^1$H NMR with that of the literature.\textsuperscript{131} Purification by silica gel column chromatography (4:1 CH$_2$Cl$_2$/ethyl acetate) yielded 2,3,4,6-O-Ac-Man ($R_f = 0.3$). Phosphitylation of 2,3,4,6-O-Ac-Man and subsequent oxidation to give the known compound dibenzyl 2,3,4,5-tetra-O-acetyl-β-D-mannopyranosyl phosphate 18\textsuperscript{111} was carried out using the standard procedure. Phosphate 18 was purified by silica gel chromatography (solvent gradient of 100% CH$_2$Cl$_2$ to 4:1 CH$_2$Cl$_2$/ethyl acetate, β-anomer 18 $R_f = 0.2$, α-anomer $R_f = 0.3$ in 4:1 CH$_2$Cl$_2$/ethyl acetate). The identity of the β-anomer 18 was confirmed by the observation of a strong NOE between H5 and H1 that was not observed for the α-anomer. $^1$H and $^{31}$P NMR spectra are reported here as the full spectral details were not reported in the literature.\textsuperscript{111} $^1$H NMR (CDCl$_3$): δ 7.34 (m, 10H, Ph), 5.45 (s, 1H, H2), 5.44 (d, 1H, $J_{1,p} = 11.3$ Hz, H1), 5.24 (dd, 1H, $J_{3,4} = 9.8$ Hz, $J_{4,5} = 9.8$ Hz, H4), 5.10-5.02 (m, 5H, CH$_2$Ph and H3), 4.24 (dd, 1H, $J_{5,6a} = 5.7$ Hz, $J_{6a,6b} = 12.3$ Hz, H6a), 4.14 (dd, 1H, $J_{5,6b} = 2.4$ Hz, $J_{6a,6b} = 12.3$ Hz, H6b), 3.75 (ddd, 1H, $J_{4,5} = 9.9$ Hz, $J_{5,6a} = 5.7$ Hz, $J_{5,6b} = 2.4$ Hz, H5), 2.15, 2.05, 1.99, 1.98 (4s, 12H, 4 x OAc), $^{31}$P NMR (CDCl$_3$): δ -1.60 (s). The remaining synthetic steps of hydrogenolysis, deacetylation, enzymatic coupling with HldE, and purification via anion exchange chromatography were performed as described in the standard procedures to give adenosine diphospho-β-D-mannose 9: $^1$H NMR (D$_2$O): δ 8.39 (s, 1H, H8), 8.14 (s, 1H, H2), 6.03 (d, 1H, $J_{1',2'} = 6.0$ Hz, H1'), 5.12 (d, 1H, $J_{1'^{-},pH} = 8.8$ Hz, H1''), 4.64-4.70 (obscured by solvent peak, H2'), 4.41 (dd, 1H, $J_{2',3'} =$
5.0 Hz, $J_{3',4'} = 3.5$ Hz, H3'), 4.28 (dd, 1H, $J_{3',4'} = 2.8$ Hz, $J_{4',5'} = 2.8$ Hz, H4'), 4.10 (m, 2H, H5'), 3.96 (d, $J_{2',3'} = 3.2$ Hz, H2''), 3.76 (dd, 1H, $J_{5''},6a'' = 2.0$ Hz, $J_{6a'',6b''} = 12.3$ Hz, H6a''), 3.57 (dd, 1H, $J_{5'',6b''} = 6.6$ Hz, $J_{6a'',6b''} = 12.0$ Hz, H6b''), 3.54 (dd, 1H, $J_{2''},3'' = 3.2$ Hz, $J_{3''},4'' = 9.4$ Hz, H3''), 3.42 (dd, 1H, $J_{3''},4'' = 9.7$ Hz, $J_{4''},5'' = 9.7$ Hz, H4''), 3.27 (ddd, 1H, $J_{4''},5'' = 9.6$ Hz, $J_{5''},6a'' = 2.1$ Hz, $J_{5''},6b'' = 6.6$ Hz, H5''), 3.00 (q, 12H, $J = 7.3$ Hz, [HN(CH$_2$CH$_3$)$_3$]¹), 1.12 (t, 18H, $J = 7.3$ Hz, [HN(CH$_2$CH$_3$)$_3$]¹); $^{31}$P NMR (D$_2$O): δ −11.01 (d, $J_{P\alpha-P\beta} = 20.8$ Hz, Pα), −12.89 (d, $J_{P\alpha-P\beta} = 21.0$ Hz, Pβ); ESIMS m/z 610.1 [M − 2H + Na]$^-$.  

2.5.6 Incubation Experiments with H1dD (NMR Spectroscopic Assay)  

H1dD was prepared for the NMR spectroscopic assay by exchanging into deuterated buffer (pH 7.0 or 8.0, 10-50 mM deuterated potassium phosphate) by ultrafiltration as follows: 50-100 µL of H1dD solution (2-20 mg/mL) was added to an ultrafiltration device (Amicon Ultra-4, 10 000 MWCO) with 1.0 mL of deuterated buffer and the solution was centrifuged at 5000 rpm (4 °C) for 10-20 min to ~100 µL. Addition of 1.0 mL of deuterated buffer followed by centrifuging was repeated twice more, such that the enzyme was finally dissolved in ~100 µL deuterated buffer. The enzyme concentration was measured using the Bradford assay. The enzyme solution was stored at 4 °C or on ice and used within a few hours. The substrate was dissolved with the same deuterated phosphate buffer to make a solution with substrate concentration of ~2-5 mM. Enough of this substrate solution was added to an NMR spectroscopy tube to allow a spectrum to be taken (400 µL), which served as the zero time point. An appropriate volume of enzyme solution or deuterated buffer (control) was added and spectra were taken, initially every 5 min, then after progressively longer time intervals.
2.5.7 Isolation of ADP-β-D-mannuronate 10, Bis(triethylamine) Salt

Crude dismutation reaction mixtures from several incubation experiments were pooled (total of 0.017 mmol of ADP-sugars) and loaded onto a DE-52 anion exchange column equipped with a UV detector ($\lambda_{\text{obs}} = 254 \text{ nm}$). A linear gradient from 0.1 to 0.8 M triethylammonium bicarbonate buffer (pH 7.4, total volume 1 L) resulted in elution of two distinct fractions, one which eluted at ~0.25-0.35 M and one which eluted at ~0.4 M buffer concentration. The fractions were lyophilized, and it was determined that the early fraction contained ADP-β-D-mannose 9 (by comparison with the NMR and mass spectrum of a synthetic sample), and the later eluting peak is ADP-β-D-mannuronate 10 (3.9 nmol, ~66 % of theoretical yield).

$^1$H NMR (D$_2$O): $\delta$ 8.42 (s, 1H, H8), 8.17 (s, 1H, H2), 6.05 (d, 1H, $J_{1',2'} = 6.1$ Hz, H1'), 5.16 (d, 1H, $J_{1''},p_{\beta} = 8.8$ Hz, H1''), 4.65-4.75 (obscured by solvent peak, H2'), 4.43 (dd, 1H, $J_{2',3'} = 5.0$ Hz, $J_{3',4'} = 3.4$ Hz, H3'), 4.30 (dd, 1H, $J_{3'',4''} = 2.8$ Hz, $J_{4'',5''} = 2.8$ Hz, H4'), 4.12 (m, 2H, H5'), 3.98 (d, 1H, $J_{2'',3''} = 3.0$ Hz, H2'''), 3.64 (dd, 1H, $J_{3''},4'' = 9.5$ Hz, $J_{4''},5'' = 9.5$ Hz, H4'''), 3.57 (dd, 1H, $J_{2''},3'' = 3.3$ Hz, $J_{3''},4'' = 9.1$ Hz, H3'''), 3.55 (d, 1H, $J_{4''},5'' = 9.7$ Hz, H5'''), 2.95 (q, 18H, $J = 7.3$ Hz, [HN(CH$_2$CH$_3$)$_3$]$^+$), 1.12 (t, 27H, $J = 7.3$ Hz, [HN(CH$_2$CH$_3$)$_3$]$^+$); $^{31}$P NMR (D$_2$O): $\delta$ -10.89 (d, 1P, $J_{\text{Pz},p_{\beta}} = 20.1$ Hz, Pz), -12.96 (d, 1P, $J_{\text{Pz},p_{\beta}} = 20.6$ Hz, Pz); ESIMS m/z 646.0 [M - 3H + 2Na]$^-$.

2.5.8 UV Spectroscopic Observation of Transient HldD-bound NADPH

HldD was exchanged into 10 mM potassium phosphate buffer (pH 8.0) by ultrafiltration as follows: 250 μL of a 19.0 mg mL$^{-1}$ (4.7 mg) HldD solution was added to an ultrafiltration device (Amicon Ultra-4, 10 000 MWCO) with 2.25 mL of buffer and the solution was centrifuged at 6 000 rpm for 20 min to give a final volume of 250 μL. This was repeated twice more and buffer was finally added to give 1.5 mL of a final solution in which the protein
concentration was measured to be 3.08 mg/mL ([epimerase subunit] = 78 μM). A 500 μL aliquot of the HldD solution was added to a quartz cuvette and was monitored at 354 nm. After 5 min, 10 μL of a 16.9 mM solution of 8 (final conc. of 331 μM) was added with thorough mixing. After 2.5 h, 10 μL of a 1 M NaBH₄ solution was added and the resulting absorbance was immediately measured.
Chapter Three

Catalytic Bases of ADP-L-glycero-D-manno-heptose 6-Epimerase
3.1 Introduction

With the establishment of the chemical mechanism of HldD, questions of how the active site is able to accommodate this chemistry arose. As described in Chapter Two, several lines of evidence support the direct redox mechanism for HldD. In this mechanism, HldD utilizes its tightly bound NADP$^+$ cofactor to oxidize directly at C-6" to generate a ketone intermediate, which is subsequently reduced from the opposite face to generate the epimeric product. How the cofactor is able to access either face of the ketone intermediate is not immediately obvious, however, there must be a reorganization of the relative positions of the cofactor and the ketone intermediate. There are many ways that this reorganization could occur.

The possibilities for the reorganization of cofactor relative to the ketone intermediate can be divided into two broad categories: those that occur within one active site, and those that occur between two active sites (between two monomers of HldD). In the one active site model the ketone intermediate is bound in one active site throughout the epimerization, and a reorganisation of the relative positions of the cofactor and the ketone intermediate occurs without release of the ketone intermediate. In the two active site scenario the ketone intermediate is released into solution and rebinds in a new orientation. Probability dictates that the second scenario is most likely to involve two separate active sites, as it is unlikely that the ketone intermediate would rebind to the same active site once it had been released into solution.

To distinguish between the two possibilities, a crossover experiment (isotopic scrambling experiment) was performed by Dr. Alain Mayer. Dr. Mayer incubated an equimolar mixture of ADP-[6"-2H]-D,D-Hep and ADP-[7"-18O]-D,D-Hep with HldD and monitored the experiment by NMR spectroscopy and mass spectrometry. Isotopic scrambling can only occur if the ketone is released in solution, and the hydride (either $^1$H$^-$ or $^2$H$^-$) is delivered to a different ketone (Figure 3.66). Dr. Mayer observed that the rate of epimerization
dramatically exceeded the rate of generation of the unlabelled or doubly labelled substrates (crossover). Scrambling of the isotopic labels was only observed on extended incubation, approximately once every 200 turnovers. Thus release of the ketone intermediate is not an intrinsic feature of the epimerization; epimerization occurs within one active site.

Figure 3.66 Dr. Alain Mayer observed isotopic scrambling once every 200 turnovers, thus the intrinsic mechanism of epimerization is intramolecular and occurs in one active site.
Perhaps the simplest explanation of how the epimerization occurs in one active site is the two-base mechanism (Figure 3.67). In this mechanism NADPH is able to access either face of the ketone intermediate carbonyl by a simple rotation of the C-5''/C-6'' bond. This would imply that HldD has two catalytic bases involved with deprotonation/reprotonation of the C-6'' hydroxyl/carbonyl, each uniquely positioned to access one epimer. Each base operates independently, but epimerization requires both catalytic residues.

While the two-base mechanism may be the simplest mechanism for HldD, one could imagine a scenario in which more complex conformational changes permit epimerization with only one catalytic base (Figure 3.68). One catalytic base appears to be sufficient for epimerization in the related enzyme UDP-galactose 4-epimerase, GalE, as discussed in Section 1.5.1 (p. 7). GalE, however, appears to be a special case as the UDP-4-ketoglucose intermediate has a pseudo C2 axis of symmetry in the glycosyl moiety. This allows a ~180 ° rotation to achieve presentation of either face of the carbonyl to the cofactor without any major changes in the position of the cofactor or the UDP moiety (Figure 1.8, p. 11). In the case of HldD, the
ketone intermediate does not bear a pseudo C2 axis of symmetry. This does not preclude that gross repositioning of the cofactor relative to bound intermediate could permit the presentation of either face of the ketone carbonyl to the cofactor and the catalytic base in a one-base mechanism.

Figure 3.68 Possible one-base mechanism of HldD.

The goal of this chapter was to investigate the validity of the two-base hypothesis and to establish the identities of catalytic residues involved. It was reasoned that mutation of a key catalytic acid/base residue to an abasic residue should destroy the ability of HldD to catalyze epimerization, whether the one-base or the two-base mechanism is operative (Figure 3.69, A). A limitation of this approach is that by simply observing a loss in activity upon mutation of an active site residue, one cannot deduce the exact role that the residue is playing. Active site residues are often involved with crucial roles other than catalytic acid/base roles, such as structural roles essential to protein folding, for example. Thus it could be misleading to identify an active site residue as a key catalytic acid/base based solely on the observation of loss of activity. In cases where it is possible to evaluate the kinetic constants of the mutant protein, the
Michaelis constant, $K_m$, reveals if the mutant protein binds the substrate as well as wild-type enzyme. However this can be misleading as well, if the mutated residue in question is involved in controlling the electrostatic environment or hydrogen bonding networks within the active site, but is not employed as an acid/base catalyst. Ideally, to best identity a catalytic residue, a control reaction is available that verifies that the mutated enzyme is still capable of catalyzing one or more steps in the catalytic cycle.

In the case of HldD an excellent control reaction requiring only one catalytic base is dismutation of the ADP-\textit{manno}-hexodialdose 8. The aldehyde 8 closely resembles the natural substrate, and as dismutation is observed the C-6'' carbonyl must sit in close proximity to the cofactor and be able to access the catalytic bases (Figure 3.69, B). In a two-base mechanism hydrated 8 will position each of its C-6'' hydroxyl groups in close proximity to one of the two catalytic bases, while simultaneously orienting the C-6'' hydride toward the cofactor. Thus deprotonation by either base could promote oxidation. Likewise, unhydrated 8 can accept a hydride from either face of the aldehyde, as there is a catalytic residue available to protonate the oxygen when either face is suitably oriented towards NADPH. If the two-base hypothesis is correct, mutation of either catalytic base should destroy epimerase activity, but leave dismutase activity relatively unaffected. This is because there is no stereochemical requirement for dismutation; dismutation is expected to occur with one catalytic base almost as well as with two catalytic bases, assuming that the mutated residue does not have any secondary roles in catalysis.
Figure 3.69 Effect of mutation of one catalytic residue on A) epimerase activity, and B) dismutase activity.

Thus, the strategy outlined in this chapter is to identify catalytic bases of HldD by screening a series of mutants for epimerase and the dismutase activities. If the two-base mechanism is correct, two mutants with no epimerase activity but with significant dismutase activity will be identified. If the one-base mechanism is correct, all mutants with no epimerase activity will equally lack dismutase activity.

In addition, dismutation studies on a synthetically more accessible aldehyde, mannose dialdehyde 20, is described. The alternate dismutation was not used in the strategy to test the two-base hypothesis; however, this work did lead to the discovery of a more efficient synthesis of ADP-manno-hexodialdose 8, which is also described in this chapter.

3.2 Dismutation of Mannose Dialdehyde 20

Although two syntheses of ADP-manno-hexodialdose 8 have been described in Chapter Two, both syntheses proved to be low yielding and laborious. To avoid a tedious replication of
the synthesis of 8, other options were examined. One option was inspired by reports that the tightly bound NAD$^+$ of the related enzyme UDP-galactose 4-epimerase, GalE, is reduced by glucose or galactose in the presence of UMP. This observation of reactivity with a truncated version of substrate sparked our curiosity to see if similar reactivity would be observed with HldD. Specifically, the HldD-catalyzed dismutation of mannose dialdehyde 20 was investigated (Figure 3.70). It was anticipated that 20 would dismutate to form mannose and mannuronate, and that this catalysis may or may not depend upon the presence of AMP or ADP. The advantage of this would be that 20 is much easier to synthesize than ADP-manno-hexodialdose 8.

![Figure 3.70 Proposed HldD-catalyzed dismutation of mannose dialdehyde to mannose and mannuronate.](image)

Mannose dialdehyde 20 was prepared in three steps from mannose (Figure 3.71). This synthesis is based upon a published synthesis of glucose dialdehyde. The hydroxyl groups were first protected with TMS protecting groups to give a ~3:1 $\alpha/\beta$ anomeric mixture of the known compound 1,2,3,4,6-penta-$O$-(trimethylsilyl)-D-mannopyranose, with column chromatography allowing isolation of the $\alpha$-anomer that was carried forward. Treatment with Collins reagent selectively oxidized the protected primary alcohol to give aldehyde 21, which proved to be stable to column chromatography and storage at 4 °C over several days. The TMS protecting groups were then removed under mildly basic conditions to generate mannose dialdehyde 20, which selectively precipitated from methanol upon cooling at −20 °C.
The $^1$H NMR spectrum of dialdehyde 20 is complex as would be anticipated given the presence of two carbonyls in the molecule (Figure 3.72. A full $^1$H NMR spectrum is in the Appendix, Figure A.100, p. 180). It is proposed that the second carbonyl allows formation of a second hemiacetal as has been observed for galactose dialdehyde$^{117}$ and is proposed for ADP-manno-hexodialdose 8 (Section 2.3.2, p. 60). It should be noted that in the open chain form of 20 the two carbonyls are homotopic, as the Fischer projection of 20 is C2 symmetrical (Figure 3.73). Formation of a six-membered ring with either carbonyl generates two possible structures: α- and β-anomers of unhydrated pyranose 20. The second carbonyl could be hydrated to form α- and β-anomers of hydrated pyranose 20. Alternatively, a ring flip to the $^1$C4 conformer of unhydrated pyranose 20 allows the second carbonyl to form a second hemiacetal, which could form with α- or β- anomeric configuration on the furanose ring. Together with the α- and β-anomeric configurations of the pyranose ring there are four possible forms of bicyclic pyranose/furanose 20. Additionally, ring opening of the pyranose ring of bicyclic pyranose/furanose 20 would form α- and β- anomers of unhydrated furanose 20, which upon addition of water could then form α- and β- anomers of hydrated furanose 20. It is also possible that a second furanose ring could form, giving bicyclic furanose/furanose 20. Precedent of the bicyclic furanose forms of 20 is the behavior of D-mannuronic acid to spontaneously form D-mannofuranurono-6,3-lactone upon crystallization.$^{135}$ There are three bicyclic furanose/furanose 20 forms possible, two of which bear a C2 axis of symmetry. Thus, 20 could
exist as a complex mixture of 15 interconverting forms in aqueous solution, and as such a full structural analysis of the $^1$H NMR spectrum of 20 was not attempted.

Figure 3.72 Partial $^1$H NMR spectrum (the ‘anomeric region’) of 20 suggests that 20 exists as a complex mixture in aqueous solution (400 MHz, D$_2$O).

Figure 3.73 Possible forms of 20 in aqueous solution.
Incubation of 20 with HldD in the presence of AMP was monitored by $^1$H NMR spectroscopy (Figure 3.74). After a 21 h incubation at 37 °C the complex signals assigned to 20 in the δ 4.8-5.5 ppm region were absent in the spectrum of the HldD-containing sample but remained in the spectrum of the control. New signals were observed in the HldD-containing test sample. Addition of authentic mannose enhanced some of these new signals, positively identifying them as coming from mannose. The remaining signals are consistent with those reported for mannuronate, and their approximate 1:1 ratio with the mannose signals is consistent with dismutation of 20. To confirm the assignments, the product mixture was separated by anion exchange chromatography, and the mannose and mannuronate products were identified by mass spectrometry and $^1$H NMR spectroscopy.

Figure 3.74 Dismutation of mannose dialdehyde 20 monitored by $^1$H NMR spectroscopy (400 MHz). Top spectrum: Control containing 18.4 mM AMP and 8.5 mM 20 in 100 mM potassium phosphate pH 8.0 deuterated buffer after 21 h incubation at 37 °C. Middle spectrum: Test reaction with 75 μM HldD after 21 h incubation at 37 °C. Bottom spectrum: Test reaction after incubation with 1.8 μmol mannose added (enhancing mannose concentration by 3.7 mM).
In a second experiment the necessity of AMP in the dismutation of 20 was investigated. Either ATP, ADP, AMP, or no nucleotide was incubated with 20 and HldD in four parallel reactions, which were monitored by \(^1\)H NMR spectroscopy. After 21 h at 37 °C the vessel containing AMP had undergone \(\sim10\%\) conversion of 20 to mannose and mannuronate, while no dismutation was observed in the other three incubations. The lack of activity in the vessel lacking any nucleotide suggests that binding of a nucleotide to HldD is required for catalytic activity. This is consistent with the literature known UMP-dependent reactivity of glucose with the tightly bound NAD\(^+\) cofactor in UDP-galactose 4-epimerase (GalE).\(^{26}\) In the case of GalE, it is thought that UMP binding initiates a conformational change in the enzyme that renders the cofactor more reactive.\(^{137}\) This is thought to arise by forcing a lysine residue closer to the positively charged nicotinamide ring upon binding of nucleotide.\(^{32,\ 138}\) It is reasonable that a similar scenario could occur with the related enzyme HldD, explaining why dismutation of 20 occurs in the presence of AMP, but not in its absence. The lack of activity in the vessel containing ADP (or ATP) suggests that the second phosphate interferes with the catalytic activity of HldD. This could be due to steric interference of the binding of 20 to the ADP-HldD complex, as the simultaneous binding of ADP and 20 to HldD forces two oxygen atoms into the same space. These observations suggest 20 binds to HldD simultaneously with AMP, mimicking ADP-\(\beta\)-d-\(\alpha\)-manno-hexodialdose 8 (Figure 3.75).

![Figure 3.75](image)

Figure 3.75 Mannose dialdehyde 20 and AMP may bind simultaneously to HldD, mimicking ADP-\(\beta\)-d-\(\alpha\)-manno-hexodialdose 8 in the active site.
The observation of the AMP-dependent dismutation of mannose dialdehyde 20 offered an alternative dismutation for use in the testing of the two-base hypothesis. The dismutation of 20, however, is less attractive as a control reaction than that of ADP-manno-hexodialdose 8 in the following aspects. First, the complex nature of the $^1$H NMR spectrum of 20 makes it difficult to quantify the conversion of 20 into mannose and mannuronate by NMR spectroscopy. As a UV/Vis spectroscopic assay is not available, the quantification of this dismutation is somewhat problematic. More importantly, however, is the fact that 20 is not covalently bonded to an ADP-moiety, raising concern that 20 may not bind to the active site in the same manner as the natural substrate. It would be reasonable to expect that 20 might have more freedom of movement within the active site than the natural substrate. This might allow 20 to access basic residues not available to the natural substrate, and allow dismutation to proceed despite the mutation of the true catalytic residue. In this respect, dismutation of 8 is a better control reaction for testing the two-base hypothesis.

The discovery of the AMP-dependent dismutation of 20 was not without merit. It prompted the investigation of the substrate specificity of the kinase activity of the bifunctional enzyme HldE, and as discussed in the next section this led to a concise, four-step chemoenzymatic synthesis of 8.

### 3.3 One Pot HldE-catalyzed Production of ADP-β-D-mannosyl

**Sugar Nucleotides from Mannosyl Sugars**

The biosynthetic strategy that *E. coli* uses to synthesize D,D-Hep 1P from D,D-Hep 7P involves the kinase activity of HldE, and the phosphatase GmhB (Figure 3.76 A). This strategy is somewhat unusual, since what is effectively a net transfer of a phosphate from C-7 to C-1 requires two enzymes. Classically in sugar-nucleotide biosynthesis similar transformations are
accomplished by a single enzyme (a mutase) without using a full equivalent of ATP (for example, phosphoglucomutase). As the phosphorylation of D,D-Hep 1P is ATP-dependent, this prompted the proposal that the kinase activity of HldE might accept mannose in the presence of phosphate buffer (Figure 3.76 B). In this strategy both mannose and phosphate are proposed to bind simultaneously to the active site of HldE, mimicking D,D-Hep 7P. This strategy challenges the notion that the C-7 phosphate of D,D-Hep 7P must be chemically bonded to the sugar moiety of substrate, and that the C-7 methylene is required for kinase activity of HldE. As the anticipated product mannose 1-phosphate 19 is known to be a substrate for the adenylyltransferase activity of HldE, ADP-mannose 9 would be the expected final product. Thus, if successful, D-mannose could be converted into ADP-mannose 9 in a one-pot reaction with a single enzyme.

Figure 3.76  A) The natural substrate for the kinase activity of HldE is D,D-Hep 7P. B) It is proposed that HldE may accept mannose for the kinase activity when phosphate buffer is present. This would generate mannose 1-phosphate 19 which is known to be accepted by the adenylyltransferase activity of HldE. ADP-mannose 9 would be made from D-mannose by HldE without phosphatase GmhB.
The incubation of mannose and ATP with H1dE in the presence of phosphate buffer was monitored by $^{31}$P NMR spectroscopy. The signals of ATP and ADP are easily distinguished by their unique chemical shifts and strong phosphorous/phosphorous coupling, as are the signals of ADP-mannose 9 at $\delta$ -11 and -13 ppm. This allows the progress of the reaction to be monitored noninvasively. Upon extended incubation of 25 mM mannose with 55 mM ATP and 30 µM H1dE in 200 mM pH 7.0 potassium phosphate, the distinctive signals of both 9 and ADP were observed in an approximate 1:1 ratio. The extent of conversion of mannose into 9 was 26 % after 13 h, which increased to 54 % after 44 h (calculated by integrating signals of ATP and 9). The product 9 was purified by anion exchange chromatography and its identity was confirmed by $^1$H and $^{31}$P NMR spectroscopy and mass spectrometry, confirming that mannose is accepted by the kinase activity of H1dE despite differing significantly from the natural substrate (Figure 3.76).

In a second experiment the concentration of phosphate was varied to determine the minimum concentration required for H1dE activity. This was important as phosphate partially co-elutes with ADP-mannosyl sugars during anion exchange chromatography. Thus in four parallel incubations the concentration of potassium phosphate was varied (200, 100, 50 and 0 mM, all pH 7.5, with 50 mM triethanolamine buffer used in the latter vessel), while the concentrations of all other components were identical. After incubation at 37 °C for 22 h the conversion of mannose to 9 was determined to be 40, 53, 65 and 81 % in the vessels containing 200, 100, 50, and 0 mM phosphate, respectively. This observation that no phosphate is required for the kinase activity of H1dE is somewhat surprising as phosphates are normally thought to be important recognition elements in many enzymatic reactions. In this case, however, the phosphate does not seem to be essential for activity. In fact, the trend indicates that free phosphate actually inhibits the activity of H1dE, however the origin of this phenomenon is not
clear. This could be an effect of ionic strength, which was not moderated in the four vessels. Alternatively, it is possible that phosphate is a weak competitive inhibitor of HldE, as there are expected to be several phosphate binding sites in the kinase and adenylyltransferase active sites (that play a role in binding ATP). Regardless of the origin of this result it is clear that phosphate is not required for HldE activity, and as such triethanolamine buffer was used in all subsequent incubations as it is easily separated from ADP-mannosyl products by anion exchange chromatography.

Encouraged by these results, mannose dialdehyde 20 was incubated with HldE. It was anticipated that 20 would be accepted by HldE, and this would allow easy preparation of the important compound, ADP-manno-hexodialdose 8. Fortunately, HldE was found to accept 20 as a substrate and 8 was produced on a similar time scale as ADP-mannose 9 had been. This promiscuous substrate specificity of HldE greatly simplifies the synthesis of 8, by installing the problematic β-mannosyl phosphate with an enzyme and shortening the synthesis of 8 to just four steps, down from eleven steps in the two previous syntheses (Figure 2.48, p. 57 and Figure 2.54, p. 63).

The substrate specificity of HldE was further probed (Table 3.1). First, the readily available epimers of mannose were investigated. The d-aldoses glucose, altrose and talose differ from mannose by the stereochemistry at C-2, C-3 and C-4, respectively. Incubation of these sugars with HldE for three days at 37 °C did not generate any ADP-sugar product nor ADP as determined by 31P NMR spectroscopy, however, addition of mannose on the third day generated ADP-mannose in high yield after only five hours. This indicates that none of these epimers of mannose are tolerated by HldE.
Table 3.1 Sugars tested for the one pot HldE-catalyzed production of ADP-β-D-mannosyl sugar nucleotides. a Test for the acceptance of the sugar by HldE is based on the three day incubation described in the text. b 2-Deoxy-2-fluoromannose was prepared by fellow lab member Pavel Glaze, but tested by the author. c Mannuronate was present in a mixture supplied by Dr. Jay Read, tested by the author. d 7-chloro-7-deoxyheptose (mixture of C-6 epimers) was prepared and tested by Dr. Alain Mayer.

As the β-linked ADP group is notoriously susceptible to intramolecular attack by the C-2" hydroxyl, generating cyclic mannose-1,2-phosphate and AMP (Section 2.2.1, p. 44), HldE-catalyzed synthesis of a stable C-2" derivatized version of substrate was attempted. Unfortunately, neither 2-deoxymannose, 2-deoxy-2-fluoromannose, 2-amino-2-deoxymannose, nor N-acetylmannosamine proved to be substrate as determined by the three-day incubation test outlined above.

It appears that only variations at C-6 of mannosyl sugars are tolerated by HldE, however the extent to which various functionalities are tolerated was unknown. It was sought to further establish the substrate tolerance of HldE to investigate the use this substrate promiscuity of
HldE as a tool to make other substrate analogs for use in the study of HldD. To this end, 6-azido-6-deoxymannose appeared to be the most accessible C-6 derivatized version of mannose available that bears a functionality larger than the C-6 hydroxyl of mannose or the C-6 aldehyde of mannose dialdehyde. The azide was prepared in two steps via a known preparation via tosylated mannose, and upon incubation with HldE two doublets characteristic of sugar nucleotides appeared in the $^{31}$P NMR spectrum. Purification of the product and characterization by $^1$H NMR spectroscopy and mass spectral analysis confirms the identity of ADP-6'-azido-6''-deoxy-β-D-mannose.

Since this work, other compounds have been investigated as substrates for HldE. Dr. Jay Read, in an attempt to prepare the ketone intermediate (Figure 2.65, p. 75), attempted to prepare the appropriate monosaccharide precursor, 6-keto-D-manno-heptose, however, was unable to purify 6-keto-D-manno-heptose, or provide conclusive evidence that 6-keto-D-manno-heptose had been made. Unfortunately, incubation of a crude product mixture did not generate the expected ketone intermediate; however ADP-mannuronate was produced in small quantities and purified. $^1$H NMR spectroscopy and mass spectrometry confirmed the identity of this product. Mannuronate is a side-product in an earlier synthetic step, and it appears to have been inadvertently carried forward during the synthesis. The observation of ADP-mannuronate formation extends the substrate specificity of HldE to include C-6 $sp^2$ hybridized sugars. In later work, Dr. Alain Mayer demonstrated that HldE accepts at least one of the epimers of 7-chloro-7-deoxyheptose to produce the corresponding ADP-sugar. It is not clear which epimer was accepted by HldE as a mixture of epimers had been used in the incubation, however it is likely 7-chloro-7-deoxy-D,D-heptose, as it has the same C-6 stereochemistry as the natural substrate.
The substrate promiscuity of the kinase activity of HldE appears to be restricted to C-6 derivatives of mannose. Fortunately, C-6 derivatives of mannose are completely relevant to the study of the epimerase HldD, and this fortunate discovery has greatly facilitated the preparation of mechanistic probes. This synthetic strategy was not used in the preparation of the natural substrate, however, as a more direct method for the preparation of ADP-D,D-Hep has been pioneered by Dr. Jay Read. With ADP-\textit{manno}-hexodialdose 8 readily available by this new four-step synthesis, and ADP-D,D-Hep provided by Dr. Jay Read and Dr. Raef Ahmed, all the substrates required for testing of the two-base hypothesis were in hand.

3.4 Site-directed Mutagenesis and Preparation of Mutants

To test the two-base hypothesis conservative mutations were introduced into HldD that converted active site acid/base residues into abasic residues. The candidate residues were identified using the available crystal structure of HldD. As discussed in Chapter One (Section 1.8.1, p. 32), the only available crystal structure of HldD has ADP-\textit{\alpha}-D-glucose bound. ADP-\textit{\alpha}-D-glucose is a poor substrate mimic, as it differs significantly from the natural substrate in the stereochemistry at C-1" and C-2" and also lacks the C-6" hydroxymethylene group. As such, the glucose moiety is not well resolved in the crystal structure, and was only modeled in three of the ten active sites of the asymmetric unit. Further, the glucose moiety is bound in two significantly different orientations in these three cases and it is therefore difficult to predict how the true substrate would bind to the active site. Nevertheless, the glucose moiety is close to the nicotinamide moiety of the NADP$^+$ cofactor, so that the general location of the active site can be deduced from this information (Figure 3.77). Close to the glucose moiety and the nicotinamide moiety of NADP$^+$ is the conserved residue tyrosine 140. This residue, along with serine 116 and lysine 144 comprise the conserved SDR catalytic triad, as determined by
sequence homology and structural information. Tyrosine 140 almost certainly serves as one of the catalytic acid/base residues, and thus was targeted for site-directed mutagenesis. Other acid/base residues positioned within 10 Å of the C-4' position of NADP⁺ were identified: lysine 178, lysine 208 and aspartate 210. Thus, the following mutant versions of HldD were prepared: Y140F, K178M, K208M and D210N. Following initial evaluation of the single mutants, a double mutant Y140F/K178M was also prepared.

Preparation of mutants was accomplished using the QuikChange Site-Directed Mutagenesis Kit from Stratagene, as described here. This mutagenesis kit relies on the fact that plasmids isolated from E. coli cells are methylated at the N-6 position of deoxyadenosine residues (m6A). This feature distinguishes the parental DNA from DNA made during PCR amplification and enables one to digest the parental DNA using the restriction enzyme Dpn I.
Dpn I cleaves the parental DNA at the target sequence 5'-Gm^6ATC-3', leaving the newly amplified DNA intact. By using PCR primers encoding the desired mutation, the newly synthesized plasmid encodes the mutant; however, it contains staggered nicks. The nicked plasmid is then transformed into *E. coli* cells where it is ligated, replicated and then isolated. Purified mutant plasmids were finally sequenced at the Nucleic Acid Protein Service (NAPS) Unit at UBC to verify no errors were introduced in the mutant genes.

Overexpression of the mutant *hldD* genes and isolation of the resulting mutant proteins was initially carried out as with wild-type HldD. Overexpression at 37 °C and purification at room temperature of Y140F, K208M and D210N mutants gave protein yields approximately equal to that of wild type (~20 mg/L culture), however the yield of the K178M mutant under these conditions was much lower (~500 μg/L culture). To improve the yield of the K178M mutant, cells expressing this mutant were grown to midlog phase at 37 °C, then moved to 23 °C upon induction and grown to OD<sub>600</sub> ~1.6. Protein purification at 4 °C gave improved yields (~15-20 mg/L culture). The double mutant Y140F/K178M, however, was overexpressed in poor yield (~500 μg/L culture), even under these cooler growth and isolation temperatures. To overcome this, the following adjustments were made. The cells were grown to midlog phase at 23 °C, then induced at 18 °C and grown to OD<sub>600</sub> ~1.6. Protein purification at 4 °C yielded ~10-15 mg/L culture of the double mutant.

Having successfully overexpressed the mutant epimerases, correct folding was assessed by determining their ability to bind NADP(H) cofactor. As discussed in Section 2.3.5 (p. 71) wild-type HldD tightly binds one equivalent of cofactor per subunit, and the affinity is sufficiently strong that the cofactor is fully retained upon purification of the enzyme. Purified recombinant HldD contains a mixture of NADP<sup>+</sup> and NADPH, as indicated by the presence of an absorption band due to the bound NADPH (Figure 2.62, p. 72). The ability of HldD to bind
cofactor reflects its tertiary structure, thus this readily observable property of the mutants was measured. The wild-type, Y140F, K208M, and D210N mutants were found to have identical UV profiles, indicating a normal ratio of NADPH bound to these proteins. In contrast, the UV spectra of the K178M and Y140F/K178M mutants conspicuously lacked the NADPH band, indicating a lack of cofactor bound to these mutants. This was confirmed upon addition of sodium borohydride, which did not generate the 354 nm band in the spectra of K178M or Y140F/K178M, whereas addition of sodium borohydride to wild-type, Y140F, K208M and D210N noticeably enhanced the bound NADPH band. Evidently K178M and Y140F/K178M had been isolated as apoenzymes, which may explain the problems of isolation with these mutants discussed above. Fortunately, incubation of these two mutants with 200 μM NADP⁺ (or NADPH) for 30 min at room temperature, followed by buffer exchange via centrifugal filtering (~1000-fold dilution of unbound cofactor), and sodium borohydride reduction gave proteins with UV spectral profiles identical to the reduced Y140F, K208M, D210N mutants and wild-type. These observations indicate that while K178M and Y140F/K178M are isolated as apoenzymes, upon reconstitution with NADP⁺ these mutants tightly bind the cofactor. While binding of the cofactor implies that the mutants are properly folded, a more convincing assay involves measurement of catalytic activity, which is addressed in the next section.

3.5 Evaluation of Epimerase and Dismutase Activities of Mutants

A sensitive assay to evaluate the epimerization reaction proved to be unattainable. Ideally, a UV/Vis spectroscopic assay is desired to monitor enzyme kinetics. However, no such assay is available for this epimerization reaction. HPLC is a common alternative for following enzyme kinetics, however, attempts to establish an HPLC assay for the epimerization were unsuccessful as baseline separation of the two epimers was not attained. ¹H NMR spectroscopy,
however, has proven to be a successful technique to monitor the epimerization. $^1$H NMR spectroscopy is not as sensitive as UV/Vis spectroscopy or HPLC, nor are the quantitative properties of NMR spectroscopic integration of the same accuracy. However, for the observation of large differences in activities (on the scale of orders of magnitude) NMR spectroscopy provides an attractive assay. As the mutation of a key catalytic residue would be expected to have a dramatic effect on catalytic activity of HldD, $^1$H NMR spectroscopy was used to screen the epimerase activities of the mutants.

The $H_5''$ signals in the $^1$H NMR spectra of the two epimers are well separated from each other and all other signals, and are therefore excellent for monitoring the epimerization (Figure 3.78). Thus, the epimerase activities were measured in NMR spectroscopy tubes using 2.7 mM ADP-D,D-Hep, far in excess of the wild-type value of $K_m$ (0.1 mM). Under these conditions the first 15% of conversion was measured by integrating $H_5''$ signals, and it was determined the His-tagged wild-type HldD (2.65 $\mu$M) exhibited a specific catalytic activity of $2.7 \pm 0.8 \mu$mol min$^{-1}$ mg$^{-1}$ at 23 °C. As can be observed in Figure 3.78, part A, the epimerization under these conditions was observed to occur over several tens of minutes; at the fastest rate possible that would still allow the reaction to be monitored by NMR spectroscopy. Under identical conditions the epimerization catalyzed by the Y140F mutant occurred at a dramatically slower rate. In this way the activities of all the single mutants and wild-type HldD were measured, and the normalized epimerase activities are 0.08 (Y140F), 0.01 (K178M), 85 (K208M), and 90% (D210N, where wild-type HldD epimerase activity = 100%). The relative epimerase activities of Y140F and K178M are three orders of magnitude lower than that of wild-type, suggesting that tyrosine 140 and lysine 178 are both important catalytic residues. In contrast, the K208M and D210N mutants retain almost all epimerase activity. Clearly lysine 208 and aspartate 210 are not important for catalysis.
Figure 3.78 Partial $^1$H NMR spectra during the epimerization of 2.7 mM ADP-D,D-Hep to ADP-L,D-Hep (400 MHz, 10 mM potassium phosphate, pH 7, 23 °C, D$_2$O). A) Epimerization catalyzed by 2.65 μM wild-type HldD. B) Epimerization catalyzed by 2.65 μM Y140F HldD.

To distinguish between whether tyrosine 140 and lysine 178 are, in fact, the catalytic bases in a two-base mechanism, or if they serve some other important function, the dismutase activities of Y140F and K178M was compared with that of wild-type HldD. In this case HPLC was an excellent assay for dismutase activity as the additional negative charge on ADP-mannuronate 10 allowed easy separation from ADP-manno-hexodiallode 8 and ADP-mannose 9 (Figure 3.79). Employing a stopped assay, incubations were carried out using 1 mM ADP-manno-hexodiallode 8 and the first 5 % of conversion was assessed. In this manner, His-tagged wild-type HldD was found to have a dismutase specific activity of $0.20 \pm 0.04 \mu$mol min$^{-1}$ mg$^{-1}$ at 37 °C. Increasing the concentration of 8 to 2 mM did not increase this activity, demonstrating that the substrate concentration is saturating. Observation of Y140F and K178M dismutase activities reveals that these mutants retain 20 and 5 % dismutase activity, respectively, relative to wild-type HldD. These activities are only five-fold and 20-fold lower than that of wild-type, showing that the mutants have retained significant levels of dismutase activity when compared to the dramatic reductions in the observed epimerase activities. This is consistent with dismutation requiring only one catalytic base. To confirm this, the double mutant
Y140F/K178M dismutase activity was assessed by HPLC. As anticipated, no dismutase activity was observed for the double mutant, consistent with the dismutase activities observed for Y140F and K178M reflecting the catalytic activity of mutants with one catalytic residue.

![HPLC chromatograms](image)

Figure 3.79 HPLC chromatograms of time points taken during the dismutation of ADP-manno-hexodialdose 8 (injected: 50 μL of 1 mM ADP-sugar solution). A) ADP-manno-hexodialdose 8 control incubated for 2 h at 37 °C. B) Product mixture after 60 min (37 °C) incubation with WT HldD. C) Product mixture after 2 h (37 °C) incubation with Y140F HldD.

The epimerase and dismutase activities measured are summarized in Table 3.2. The data is consistent with the two-base hypothesis: two mutants with severely compromised epimerase
activity retain significant dismutase activity. One might expect the Y140F and K178M mutants to retain exactly 50% of the wild-type dismutase activity in theory, but this would assume that each catalytic residue operates in entirely independent fashion and does not interact with other residues in the enzyme. This ideal scenario does not appear to be the case, nor would one realistically expect that to be the case as a charged residue in the active site is likely to be involved in hydrogen-bonding and electrostatic interactions with other residues. Interrupting those interactions by replacing the catalytic residue with an uncharged residue such as phenylalanine or methionine evidently carries a price on the catalytic activity. Fortunately this price is not so severe as to destroy dismutase activity entirely in the single mutants, jeopardizing any meaningful conclusions. As it is, the data appears to be consistent with the two-base mechanism, and identifies the two catalytic acid/base residues as tyrosine 140 and lysine 178.

<table>
<thead>
<tr>
<th>H1dD</th>
<th>Epimerase Activity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Dismutase Activity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Y140F</td>
<td>0.08</td>
<td>20</td>
</tr>
<tr>
<td>K178M</td>
<td>0.1</td>
<td>5</td>
</tr>
<tr>
<td>K208M</td>
<td>85</td>
<td>n.d.&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>D210N</td>
<td>90</td>
<td>n.d.&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Y140F/K178M</td>
<td>&lt; 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 3.2 Summary of epimerase and dismutase activities for H1dD mutants relative to wild-type. <sup>a</sup> Versus His-tagged wild-type H1dD. <sup>b</sup> Not determined. <sup>c</sup> Product not observed (estimated maximum possible activity).

### 3.6 Conclusions and Future Work

This chapter summarizes the advances made in the understanding of how the H1dD active site is able to accommodate the chemical mechanism of epimerization. The work of Dr. Alain Mayer demonstrates that epimerization occurs within one active site. Dr. Mayer found that two pools of isotopically labeled substrates epimerize independently from each other.
Scrambling was only observed upon extended incubation and was correlated with release of the ketone intermediate, occurring once every 200 turnovers. Thus, in the intrinsic mechanism the ketone intermediate does not leave the active site, and the tightly bound NADPH is able to access either face of the ketone intermediate.

How this nonstereospecific hydride transfer occurs in one active site was addressed in this chapter. Two hypotheses were proposed, the two-base mechanism (Figure 3.67, p. 97) and the one-base mechanism (Figure 3.68, p. 98). To discern between these two mechanisms a series of mutants was screened for epimerase activity, and two mutants were identified as having epimerase activities more than three orders of magnitude lower than the wild-type. The dismutase activities of Y140F and K178M, however, were measured to be only five-fold and 20-fold lower than wild-type, respectively. The discrepancy between the epimerase and dismutase activities of these mutants suggests that Y140F and K178M each retain one catalytic base. Consistent with this, the double mutant Y140F/K178M displays no dismutase activity. These results suggest HldD requires two catalytic bases for epimerization, and identify those residues as tyrosine 140 and lysine 178.

The mechanism of HldD, with tyrosine 140 and lysine 178 shown playing the roles of the two catalytic acid/base residues, is summarized in Figure 3.80. While the work described in this chapter implicates tyrosine 140 and lysine 178 as the acid/base residues required for catalysis, it does not distinguish which residue deprotonates the hydroxyl group of a given epimer. For illustrative purposes, stereospecific roles of the two residues have been shown here. An inspection of the crystal structure suggests that lysine 178 deprotonates ADP-\(\text{D, D-Hep}\) tyrosine 140 deprotonates ADP-\(\text{L, D-Hep}\)^{90} however, given the disordered nature of the bound sugar nucleotide, it is a speculative assignment.
Figure 3.80 Two-base mechanism of HldD with tyrosine 140 and lysine 178 playing the roles of catalytic acid/base residues. The stereospecific roles shown here for illustrative purposes are based upon examination of the crystal structure. Further studies are required to make this distinction.

The observed role of tyrosine 140 is not surprising given this is the conserved tyrosine of the catalytic triad (which also includes serine116 and lysine 144). As discussed in Chapter One (Section 1.4, p. 5), the role of the conserved tyrosine in other SDR enzymes is to act as the catalytic base that removes the hydroxyl group proton during oxidation of an alcohol (or protonates the carbonyl during a reduction step).

The observed role of lysine 178 is somewhat unusual amongst SDR family members, as a residue at this position is not normally involved in hydride transfer steps. However, with one other SDR family member, GDP-mannose 3,5-epimerase, GME (Section 1.5.3, p. 17), it is suggested that a lysine in an analogous position serves as a catalytic acid/base residue to promote the deprotonation at C-3" or C-5" to form enolate intermediates (Figure 1.16, p. 19).

In future studies, the stereospecific roles of tyrosine 140 and lysine 178 will be addressed. According to the two-base mechanism (Figure 3.67, p. 97), each catalytic base is uniquely responsible for deprotonating the C-6" hydroxyl of one epimer. The identities of the
epimer that each catalytic base operates on will be deduced by the following strategy (Figure 3.81). The ketone intermediate will be incubated with one equivalent of either Y140F or K178M with its cofactor in the reduced state. According to the two-base mechanism, each mutant should stereospecifically reduce the ketone intermediate, with each mutant generating a unique epimer. The role of the remaining catalytic base in the mutant is to deprotonate the C-6” hydroxyl of the observed epimer.

![Figure 3.81 Strategy to identify stereospecific roles of each catalytic base.](image)

The above experiment requires the synthesis of the ketone intermediate as well as the preparation of one equivalent of mutant HldD with reduced cofactor. To avoid using a full equivalent of mutant HldD with reduced cofactor, ADP-*manno*-hexodialdose 8 may prove to be useful by reducing the tightly bound NADP\(^+\) cofactor in situ (Figure 3.82). As 8 is known to quickly reduce the tightly bound cofactor to NADPH (Figure 2.63, p. 73), the ketone intermediate, which is expected to exist primarily in its unhydrated form, may be able to intercept the reduced epimerase. This would allow catalytic amounts of each mutant HldD to be used, and the progress of the reaction could be monitored by \(^1\)H NMR spectroscopy, which would be difficult in the strategy described in Figure 3.81.
Figure 3.82 Catalytic strategy to identify stereospecific roles of catalytic bases using ADP-manno-hexodialdose 8 to reduce tightly bound NADP⁺ in situ.

The crystal structure of HldD with an appropriate β-linked mannosyl sugar nucleotide bound is also being pursued with collaborators at the University of Saint Andrews. A crystal structure with substrate (or at least with a better substrate mimic than ADP-α-glucose) bound may yield insight as to the actual conformational changes that occur during catalysis. The two-base mechanism predicts that each epimer binds in a manner differing by the rotation of the C-5"/C-6" bond. Ideally two crystal structures with the two different epimers in two distinct binding modes will be solved. Each epimer would position its C-6" hydride towards the cofactor and its C-6" hydroxyl towards the appropriate catalytic base.

3.7 Experimental

3.7.1 Materials and General Methods

Chemicals were purchased from Sigma-Aldrich and used without further purification unless otherwise noted. Dry methylene chloride and pyridine were distilled fresh using CaH₂ as drying agent. Recombinant hexahistidine-tagged enzymes HldD and HldE (genes cloned previously from Escherichia coli K-12 W3110) were prepared with the aid of the pET-30 Xa/
LIC vector kit (Novagen) as described in Section 2.5.2, p. 76 and Section 2.5.3, p. 77. Inorganic pyrophosphatase (from yeast) was purchased from Roche Diagnostics Corporation, and alkaline phosphatase (from bovine calf intestine) was purchased from Sigma. $^1$H NMR spectra were acquired on Bruker AV300 and AV400 instruments at field strengths of 300 or 400 MHz. Proton-decoupled $^{31}$P NMR spectra were obtained at 121.5 or 162 MHz. Mass spectrometry was performed by electrospray ionization (ESIMS) using a Waters Micromass LCT mass spectrometer. HPLC was performed using a Waters 600 instrument with a Waters 2996 photodiode array detector. The substrate ADP-D,D-Hep was prepared as described previously.$^{124}$

### 3.7.2 Standard Procedures

#### 3.7.2.1 Preparation of ADP-β-D-mannosyl Sugar Nucleotides from Mannosyl Sugars using HldE Kinase and Adenylyltransferase Activities

A 50 mM solution of the mannosyl sugar in water (with 20 % D$_2$O for NMR spectroscopy locking purposes) containing 110 mM ATP, 5 mM MgCl$_2$ in 200 mM pH 7.5 triethanolamine was prepared, to which an equal volume of a solution containing 50 μM (2.9 mg/mL) HldE and 10 μg/mL of inorganic pyrophosphatase was added, and the resulting solution was incubated at 37 °C and monitored by $^{31}$P NMR spectroscopy over several days. The growth of the ADP-mannosyl product signals at approximately -10 and -12 ppm were monitored, as was the cyclic mannosyl-1,2-phosphate singlet at 18 ppm. As cyclic mannosyl-1,2-phosphate is a product of the decomposition of ADP-β-mannosyl compounds which also produces AMP,$^{104}$ hydrolysis of phosphate from AMP was required when the cyclic mannosyl-1,2-phosphate was observed to facilitate product isolation by anion exchange chromatography.
In this case alkaline phosphatase (to give a solution of 8 µg/mL) was added and the solution was incubated until the remaining ADP and ATP had been consumed (AMP is more difficult to identify unambiguously by $^{31}\text{P}$ NMR spectroscopy). The solution was then filtered by centrifugal filtration (Amicon Ultra-4, 10 000 MWCO) and diluted two-fold with water before loading on to an anion exchange column (DE-52 resin) equipped with a UV detector ($\lambda_{\text{obs}} = 254$ nm). The adenosine and other uncharged impurities were eluted with 0.1 M triethylammonium bicarbonate buffer pH 7.5. A gradient to 0.5 M buffer was then used to elute fractions containing product, which were pooled, frozen and lyophilized to dryness.

3.7.3 Specific Synthetic Procedures

3.7.3.1 1,2,3,4-Tetra-O-trimethylsilyl-α-D-manno-hexodialdose 21

Aldehyde 21 was prepared by Collins oxidation of known compound 1,2,3,4,6-penta-O-(trimethylsilyl)-α-D-mannopyranose, using a method developed for the gluco-epimer. The pentasilylation of mannose was carried out as follows. To a solution of 1.00 g mannose (5.60 mmol) in 5.5 mL dry pyridine, 4.25 mL chlorotrimethylsilane (33.6 mmol, 6.0 equivalents) was added at room temperature. After stirring for 6 h, 40 mL diethyl ether was added to the mixture and the organic layer was washed twice with 2 x 30 mL water and once with 30 mL brine. The organic layer was then dried over magnesium sulfate and the solvent was evaporated in vacuo to yield 2.3 g of clear, light yellow oil. A $^1\text{H}$ NMR spectrum of the crude product revealed a ~3:1 $\alpha/\beta$ anomeric mixture. The anomeric mixture was purified by silica gel column chromatography with 3:1 petroleum ether/CH$_2$Cl$_2$ to give 2.05 g 1,2,3,4,6-penta-O-(trimethylsilyl)-D-mannopyranose as a colourless oil (3.79 mmol, 68% yield, $R_f = 0.3$ (3:1 petroleum ether/CH$_2$Cl$_2$), MS $m/z$ 563.2 [M + Na$^+$]). Earlier column fractions contained one anomer, determined to be the $\alpha$-anomer by comparison with literature $^1\text{H}$ NMR data. For
ease of product identification the α-anomer was carried on exclusively in the next synthetic steps. Chromium trioxide (1.72 g, 17.2 mmol), 60 mL dry CH₂Cl₂ and 2.8 mL dry pyridine were stirred at room temperature for 30 min, then chilled on ice for 5 min. A solution of 1.55 g 1,2,3,4,6-penta-O-(trimethylsilyl)-α-D-mannopyranose (2.86 mmol) in 4 mL dry CH₂Cl₂ was added drop-wise and the reaction mixture was allowed to stir at 0 °C. After one hour the dark brown reaction mixture was filtered through silica gel, which was then rinsed with 2 x 60 mL ethyl acetate. The filtered crude material was dried in vacuo to give 1.6 g clear yellow oil. Purification by column chromatography (1:1 CH₂Cl₂/petroleum ether to 100 % CH₂Cl₂) gave 243 mg 21 as a colourless oil (0.52 mmol, 18 % yield, Rf 0.1 to 0.4 streak in CH₂Cl₂). ¹H NMR (CDCl₃): δ 9.67 (s, 1H, H₆), 5.09 (s, 1H, H₁), 4.06 (d, 1H, J₄,₅ = 5.4 Hz, H₅), 4.03 (dd, 1H, J₃,₄ = 5.6 Hz, J₄,₅ = 5.6 Hz, H₄), 3.81 (d, 1H, J₃,₄ = 4.3 Hz, H₃), 3.67 (dd, 1H J₃,₄ = 4.8 Hz, J₂,₃ = 2.2 Hz, H₂), 0.16, 0.11, 0.11, 0.10 (4 x s, 36H, (CH₃)₃Si); ESIMS m/z 521.1 [M + MeOH + Na]⁺ (hemiacetal with methanol solvent).

3.7.3.2 Mannose Dialdehyde 20

Deprotection of aldehyde 21 was carried out using a method adapted from the literature preparation of the gluco-epimer. Aldehyde 21 (308 mg, 0.660 mmol) was stirred in 5.3 mL of a 1 mg/mL K₂CO₃ in methanol solution at room temperature for one hour. Precipitation of the product by cooling to −20 °C and subsequent addition of 1:1 diethyl ether/ethyl acetate yielded 31 mg mannose dialdehyde 20 as a white powder (0.17 mmol, 26 % yield). ¹H NMR spectrum (D₂O, Figure A.101, p. 180) of 20 is complex, consistent with compounds of this type. ESIMS m/z 379.0 [2 M + Na]⁺.
3.7.3.3 Adenosine Diphosphate-β-D-manno-hexodialdose,
Bis(triethylamine) Salt 8 (From 21)

Deprotection of aldehyde 21 was carried out as described above in the preparation of 20, however in the streamlined synthetic approach (which was found to be higher yielding) the crude mixture of 20 was used in the incubation with HldE. Aldehyde 21 (119 mg, 0.255 mmol) was stirred in 1.5 mL of a 1 mg/mL K2CO3 in methanol solution at room temperature for one hour. The crude solution was diluted to 10 mL to create a solution containing 0.54 mmol ATP (2.1 equivalents), 150 mM pH 7.5 triethanolamine, 2 mM MgCl2, 2.6 mg HldE, 25 μg inorganic pyrophosphatase and 10 % D2O. The reaction mixture was incubated at 25 °C and progress was monitored by 31P NMR spectroscopy. After 24 h the solution was filtered by centrifugal filtration (Amicon Ultra-4, 10 000 MWCO) and loaded onto an anion exchange column (DE-52 resin) equipped with a UV detector (λobs = 254 nm) and eluted with a gradient of 0.1 to 0.5 M triethylammonium bicarbonate buffer pH 7.5. Fractions containing product were lyophilized and pooled. The product adenosine diphosphate-β-D-manno-hexodialdose, bis(triethylamine) salt 8 was identified by match of 1H, 31P NMR and mass spectra with an authentic sample (Section 2.5.5.7, p. 86). Quantification by UV absorbance (λobs = 254 nm) showed that 0.178 mmol of 8 was isolated (70 % yield from 21). The purity was estimated to be 95 % by HPLC.

3.7.3.4 Adenosine 5′-(β-D-manno-pyranosyl diphosphate),
Bis(triethylamine) Salt 9 (From Mannose)

Mannose (41 mg, 0.23 mmol) was incubated with HldE for six days as outlined in the standard procedure in Section 3.7.2.1. Following purification by anion exchange chromatography the product containing fractions were combined to give 0.151 mmol of ADP-β-D-mannose (66 % yield) as determined by UV measurement (λobs = 259 nm). The mass
spectrometry, $^1$H and $^{31}$P NMR spectra matched that reported in Section 2.5.5.11 (p. 90) for ADP-β-D-mannose.

### 3.7.3.5 Adenosine 5′-(6″-azido-6″-deoxy-β-D-manno-pyranosyl diphosphate), Bis(triethylamine) salt 22

From mannose, a tosyl group was installed at C-6, and in a second step the tosylate was displaced by azide to generate 6-azido-6-deoxy-mannose as previously described. The identity of 6-azido-6-deoxy-mannose was confirmed by comparison of the $^1$H NMR spectrum of the purified compound with literature data. 6-Azido-6-deoxy-mannose (42 mg, 0.205 mmol) was then incubated with HldE for 16 h as described in Section 3.7.2.1. Following anion exchange chromatography 14.7 μmol of Adenosine 5′-(6″-azido-6″-deoxy-β-D-manno-pyranosyl diphosphate), bis(triethylamine) salt 22 was isolated (7.2 % yield), as measure by UV spectroscopy ($\lambda_{obs} = 259$ nm). $^1$H NMR (D$_2$O): $\delta$ 8.51 (s, 1 H, H8), 8.25 (s, 1 H, H2), 6.14 (d, 1 H, $J_{\text{1',2'}} = 5.9$ Hz, H1'), 5.24 (d, 1 H, $J_{\text{1',p}} = 8.9$ Hz, H1''), 4.76 (d, 1 H, $J_{\text{1',2'}} = 5.8$ Hz, H2'), 4.52 (dd, 1 H, $J_{\text{2',3'}} = 4.3$ Hz, $J_{\text{3',4'}} = 4.3$ Hz, H3'), 4.39 (m, 1 H, H4'), 4.22 (m, 2 H, H5'), 4.09 (d, 1 H, $J_{\text{2',3'}} = 2.4$ Hz, H2''), 3.69-3.63 (2H, H3'' and H6a''), 3.60 (dd, 1 H, $J_{\text{3',4'}} = 9.3$ Hz, $J_{\text{4',5'}} = 9.3$, H4''), 3.54 (dd, 1 H, $J_{\text{5',6b'}} = 5.6$ Hz, $J_{\text{6a',6b'}} = 13.5$ Hz, H6b''), 3.44 (m, 1 H, H5''), 3.19 (q, 12 H, $J=7.3$ Hz, [HNCH$_2$CH$_3$)$_3$]), 1.27 (t, 18 H $J=7.3$ Hz, [HNCH$_2$CH$_3$)$_3$]); $^{31}$P NMR (D$_2$O) $\delta$ ppm $-8.94$ (d, 1 P, $J_{\text{p,p}} = 20.5$ Hz, Pα), $-11.27$ (d, 1 P, $J_{\text{p,p}} = 20.4$ Hz, Pβ); ESIMS m/z 613.2 [M – H].

### 3.7.4 Site-Directed Mutagenesis and Preparation of HldD Mutants

Mutant plasmids were prepared using the QuikChange Site-Directed Mutagenesis Kit from Stratagene. Oligonucleotide primers used are listed below, with the mutated nucleotides underlined. Primers for the Y140F mutant were 5′-
GAAAAACCGTTGAACGTCTTCGGTTACTCAAAATTCCTG-3' (forward) and 5'-
CAGGAATTTTGAGTAACCGAAGACGTTCAACGGTTTTTC-3' (reverse). Primers for the
K178M mutant were 5'-GTGAAGGCCCATATGGGCAGCATGCGAG-3' (forward) and 5'-
CTCGCCATGCTGCCCATATGGCCCTCAC-3' (reverse). Primers for the K208M mutant
were 5'-GAAGGTAAGCGAGAAGTTTCTGCTATATGTG-3' (forward) and 5'-
CACATAGACGAAGTCGCGCATGAAGCTCGCTACCTTC-3' (reverse). Primers for the
D210N mutant were 5'-GAACCTCAAACGCGATTTTCGCTATATGTGCGG-3' (forward) and
5'-CGCCCACATAGACGAAATCGCGTTTGAAGTTTC-3' (reverse). Double mutant
Y140F/K178M was prepared by mutagenesis PCR of the Y140F plasmid with K178M primers.
Mutant gene sequences were confirmed by sequencing the entire gene. Overexpression (at 37
°C) and purification (at 23 °C) of Y140F, K178M, D210M and D210N mutants and wild-type
hexahistidine-tagged H1dD was identical as wild-type (Section 2.5.3, p. 77). The K178M and
Y140F/K178M mutants were overexpressed and purified with the same protocol, only at lower
temperatures. The K178M mutant protein was grown to midlog phase at 37 °C, then induced
with isopropyl β-D-galactopyranoside (IPTG) and grown at 23 °C to OD600 ~1.6. The
Y140F/K178M double mutant was grown at 23 °C to midlog phase, then induced with IPTG
and grown at 18 °C to OD600 ~1.6. Both K178M and Y140F/K178M were purified by nickel-
affinity chromatography at 4 °C. All mutants were dialyzed, flash frozen and stored at −80 °C at
25 to 50 μM as described in Section 2.5.3 (p. 77). When desired, an aliquot of H1dD was
allowed to thaw to room temperature and NADP+ was added to give 200 μM NADP+. The
incubation with NADP+ occurred for 30 min, then buffer was exchanged to 10 mM pH 7.0
potassium phosphate (deuterated buffer for NMR experiments) by centrifugal filtering (Amicon
Ultra-4, 10 000 MWCO) at 6000 x g to affect a 1000 times dilution of cofactor. Protein
concentrations were measured using Bradford dye.
3.7.5 Incubation Experiments

3.7.5.1 Incubation of Mannose Dialdehyde 20 with HldD

An 800 μL solution containing 3.4 mg 20 (19 μmol) and 10 mg AMP (27 μmol) and 950 mg HldD (24 nmol) in 100 mM pH 8.0 deuterated potassium phosphate buffer was incubated at 37 °C. The reaction was monitored by $^1$H NMR spectroscopy until judged complete by the disappearance of signals attributed to 20. The dismutated product mixture was loaded onto an AG 1-X8 anion exchange column (BioRad) and eluted using a step gradient of two column volumes at each step. The eluents comprised of aqueous formic acid, in concentration from 0 to 1 M increasing in 0.1 M intervals, and the fractions were lyophilized to dryness. The first fraction (containing no formic acid) contained mannose, as confirmed by $^1$H NMR spectroscopy and ESIMS ($m/z$ 203.0 [M + Na]$^+$). The 0.3 M formic acid fraction contained D-mannuronic acid, as confirmed by ESIMS ($m/z$ 192.8 [M $-$ H]$^-$) and comparison of the $^1$H NMR spectrum with that in the literature.\textsuperscript{136}

3.7.5.2 Testing the Nucleotide Dependence of the HldD-Catalyzed Dismutation of Mannose Dialdehyde 20

Four NMR spectroscopy tubes containing 16.8 mM 20, 10 μM HldD, 3.2 mM of either ATP, ADP, AMP, or no nucleotide in 100 mM pH 7.0 deuterated potassium phosphate buffer were incubated for 21 h at 37 °C. The conversion of 20 to mannose and mannuronate was measured by integration of the anomeric signals in the $^1$H NMR spectra.
3.7.5.3 Testing HldE Kinase and Adenylyltransferase Substrate Specificity with Monosaccharides

The above procedure for the preparation of ADP-mannosyl sugar nucleotides (Section 3.7.2.1) was scaled to 5 mg of the sugar in question to allow for incubation in an NMR spectroscopy tube. After incubation for three days at 37 °C, if no ADP or ADP-sugar product signals were observed in the $^{31}$P NMR spectrum then 5 mg mannose was added. After incubation for 5 h at 37 °C, ADP-mannose signals were observed in the $^{31}$P NMR spectrum (typically ~20-30 % conversion), confirming that the HldE used was functional.

3.7.5.4 Measurement of Epimerase Activity by $^1$H NMR Spectroscopy

In a parallel incubation in NMR tubes the appropriate version of HldD was added to a D$_2$O solution containing ADP-D,D-Hep and 10 mM pH 7.0 potassium phosphate buffer. The tube was inverted three times to allow mixing of the 580 µL solution containing 2.7 mM substrate and 2.65 µM enzyme. Conversion of ADP-D,D-Hep to ADP-L,D-Hep was followed using both $^1$H and $^{31}$P NMR spectroscopy at 23 °C.

3.7.5.5 Measurement of Dismutase Activity by HPLC

In five 800 µL snap-top tubes aldehyde 8 in 10 mM potassium phosphate buffer pH 7.0 was equilibrated at 37 °C for 5 min. The parallel incubations were initiated by addition of the appropriate version of HldD to each tube (either wild-type, Y140F, K178M, Y140F/K178M or a control which contained no enzyme), inverting three times, and returning the tube to the incubation bath. Substrate and enzyme concentrations of the 500 µL solutions were 1 mM and 0.5 µM, respectively. At appropriate intervals (15 min for wild-type, 30 or 60 min for the mutants) 50 µL aliquots were removed and flash frozen in liquid nitrogen. Immediately prior to
injection the aliquots were thawed by addition of 50 μL room temperature deionized water, and the solution was injected within 10 s of thawing. The reversed-phase ion-pair HPLC protocol using a Waters Spherisorb ODS II 5 μm (250 x 4.6 mm) column was adapted from the literature. Two mobile phases were used: mobile phase A containing 50 mM pH 7.0 potassium phosphate, 2.5 mM tetrabutylammonium bisulfate (TBAHS) and mobile phase B containing 50 mM pH 7.0 potassium phosphate, 2.5 mM TBAHS bisulfate, 50 % acetonitrile. A linear gradient from 2.5 % to 30 % mobile phase B over 30 min at a flow rate of 1 mL/min eluted the ADP-sugars, which were detected at 259 nm. Aldehyde 8 and ADP-mannose 9 co-eluted at 19 min and ADP-mannuronate 10 eluted at 23 min.
Chapter Four
Mechanistic Studies on
UDP-N-acetylglucosamine 5-Inverting 4,6-Dehydratase
4.1 Introduction

As discussed in Chapter One, UDP-N-acetylglucosamine 5-inverting 4,6-dehydratase (PseB) is a recently discovered sugar nucleotide 4,6-dehydratase of the SDR enzyme family. PseB is unique amongst the known sugar nucleotide 4,6-dehydratases in that it inverts the configuration at C-5" during dehydration (Figure 4.83). Given that the three known dehydratases are non-inverting and have been studied extensively (Section 1.7, p. 25), it is the C-5" inversion process that is the most intriguing aspect of this reaction. The goal of this chapter is to elucidate the mechanism of this unusual dehydration.

![Proposed mechanism of PseB](image)

Figure 4.83 Proposed mechanism of PseB.

The mechanism proposed here is based upon the established mechanisms of the non-inverting dehydratases (Figure 4.83). In the first step PseB uses its tightly bound NADP⁺ cofactor to oxidize UDP-GlcNAc at C-4". This enables dehydration, generating an α,β unsaturated ketone intermediate. In the final reduction step the α,β unsaturated ketone intermediate receives a hydride from NADPH at C-6", and a proton at C-5". This mechanism
differs from that of the non-inverting dehydratases in that C-5" protonation occurs on the opposite face of the double bond, to generate a C-5" inverted product. One might expect up to four distinct residues to fulfill the four distinct catalytic acid/base roles proposed in this mechanism.

It was first sought to establish the operative mechanism of PseB. A simple experiment that was anticipated to differentiate between our proposed mechanism (Figure 4.83) and the literature proposed mechanism (Figure 1.36, p. 39), was to perform the dehydration in D_2O. According to our proposed mechanism, deuterium should be incorporated at C-5", while according to the literature mechanistic proposal it should be incorporated at C-6".

Following confirmation of the proposed mechanism, the identities of the residues that play the roles of B_3 and B_4 will be sought (Figure 4.83). The strategy is to first identify PseB-catalyzed reactions that probe individual steps of the dehydration, then to screen mutants for their ability to perform these reactions.

To identify the residue responsible for C-5" protonation during the reduction step (B_4, Figure 4.83) the wash in of deuterium at C-5" of the UDP-arabino-sugar product will be examined (Figure 4.84). Isotopic exchange into product has not been reported for PseB, but it is well described for dTDP-glucose 4,6-dehydratase (RmlB), and allowed the identification of the analogous catalytic residue in that enzyme. To identify B_4, a mutant that is unable to catalyze C-5" isotopic exchange into product, but which retains other catalytic activities is sought.

\[ \text{UDP-arabino-sugar} \xrightarrow{\text{PseB}} \text{UDP-\{5-2H\}-arabino-sugar} \]

Figure 4.84 PseB is proposed to catalyze isotopic exchange at H5" of UDP-arabino-sugar.
With the eventual goal of identifying the catalytic residue responsible for protonation of the C-6" hydroxyl during dehydration (B3, Figure 4.83), UDP-6-deoxy-6-fluoro-GlcNAc 23 will be investigated as a substrate analog (Figure 4.85). It is anticipated PseB will eliminate HF from 23, generating the UDP-arabino-sugar product in an analogous way as PseB eliminates water from UDP-GlcNAc. As fluoride is not anticipated to require protonation during elimination, the mutant lacking B3 should still catalyze turnover of 23. This strategy has proven to be successful in the study of dTDP-glucose 4,6-dehydratase (RmlB), allowing identification of the corresponding catalytic residue in that enzyme.76

![Figure 4.85](image)

Figure 4.85 PseB is proposed to eliminate HF from UDP-6-deoxy-6-fluoro-GlcNAc 23.

### 4.2 Site-Directed Mutagenesis and Preparation of Mutant and Wild-Type PseB

The source of PseB used in this work is from *C. jejuni* subspecies *jejuni* (strain NCTC 11168), which was made available by the generous donation of the plasmid pNRC20 containing the *pseB* gene by collaborators at the National Research Council of Canada. PseB from this organism shares 62.9 % sequence identity with the PseB from *H. pylori* that has been studied by X-ray crystallography.102 Most importantly, the residues implicated from the crystal structure to be key acid base catalysts are conserved. These residues are aspartate 126, lysine 127 and tyrosine 135 in *C. jejuni* PseB. In this study the following conservative mutations were chosen: D126N, K127A, and Y135F.
In preparation for site-directed mutagenesis, sequencing of the \textit{pseB} gene in the pNRC20 plasmid revealed two mutations in the nucleotide sequence, g328t and t384c. While the latter mutation is silent (does not change the expressed protein sequence), the former mutation encodes for a D110Y mutation. Thus, in the first round of mutagenesis the g328t mutation was corrected, while the t384c mutation was not addressed. This created the plasmid with the gene encoding the His-tagged wild-type PseB that was used to prepare plasmids encoding D126N, K127A, and Y135F.

Mutagenesis was accomplished as described in the directions for the QuikChange Site-Directed Mutagenesis Kit from Stratagene, using some components from other sources. A description of the strategy employed by the QuikChange Site-Directed Mutagenesis Kit can be found in Section 3.4 (p. 112). Following mutagenesis, the wild-type and mutant genes encoded on the purified plasmids were sequenced at the Nucleic Acid Protein Service (NAPS) Unit at UBC to verify that no errors were introduced.

Wild-type and mutant PseB proteins were overexpressed and purified in the same manner as described for HldD and HldE (Section 2.2.1, p. 44). The plasmid used in this work encodes for kanamycin resistance, and the expression of the \textit{pseB} genes is under the control of the \textit{lac} operon, allowing induction with IPTG. Wild-type and mutant PseB proteins all overexpressed well in \textit{E. coli} BL21(DE3) at 37 °C. The recombinant proteins contain an N-terminal His-tag, and were readily purified by nickel affinity chromatography with typical yields ~20 mg protein/L of cell culture. SDS-PAGE analysis of the purified 39 kDa recombinant proteins demonstrates that > 90 % purity was attained by this method (Figure 4.86).
Proper folding of the recombinant proteins was assessed by observation of tightly bound NADPH with UV/Vis spectroscopy. It has been reported that recombinant PseB from \textit{H. pylori} is isolated with NADP$^+$/NADPH tightly bound,\textsuperscript{102} and that PseB from \textit{C. jejuni} does not require exogenous cofactor for activity.\textsuperscript{92} As tight binding of the cofactor to purified PseB implies that the protein is correctly folded, the UV spectrum of wild-type PseB was measured. No significant absorbance was observed at wavelengths > 310 nm. Upon addition of sodium borohydride absorbance bands at 344 nm and 425 nm were observed, consistent with the generation of tightly bound NADPH. The 344 nm band is due to the biologically relevant 1,4 reduction product, and the 425 nm shoulder is due to 1,2 and 1,6 reduction products, which are known to form upon reduction of NAD$^+$ with sodium borohydride.\textsuperscript{123} Similar observations have been observed upon sodium borohydride reduction of the tightly bound NAD$^+$ of dTDP-glucose 4,6-dehydratase (RmlB).\textsuperscript{72} The observations reported here are consistent with the isolation of wild-type PseB with tightly bound cofactor mostly (> 90 \%) in the oxidized form.

UV spectral analysis of the three PseB mutants revealed a markedly different situation. The UV spectra of D126N, K127A and Y135F were all identical, exhibiting a strong absorbance band at 344 nm (Figure 4.87). Upon addition of sodium borohydride only a small
increase in absorbance was observed, indicating that ~95% of the tightly bound cofactor is in the reduced, NADPH form.

![Graph of UV spectrum](image)

**Figure 4.87** Partial UV spectrum of 128 μM D126N in 20 mM pH 9.0 Tris before and after the addition of 500 nmol sodium borohydride (to give 1.2 mM sodium borohydride).

To allow a meaningful study of the mutant dehydratases the catalytically relevant oxidized form of the tightly bound cofactor was required. To this end a reagent that would selectively oxidize the tightly bound cofactor in situ was sought. It was proposed that a substrate analog bearing a ketone at C-4" would oxidize the tightly bound NADPH as described in Figure 4.88, part A. This 4-keto substrate analog is UDP-4-keto-6-deoxy-GlcNAc, which is formed by the non-inverting dehydratase PgIF, an enzyme involved with the biosynthesis of UDP-\(N, N'\)-diacetylbacillosamine (Figure 4.88, part B).\(^{92}\)
Figure 4.88 A) In situ oxidation of tightly bound NADPH with UDP-4-keto-6-deoxy-GlcNAc. B) UDP-4-keto-6-deoxy-GlcNAc is the product of PglF-catalyzed dehydration of UDP-GlcNAc.

The dehydratase PglF was available thanks to the generous donation of the plasmid pNRC40.1 from collaborators at the National Research Council Canada. This plasmid encodes a truncated form of PglF (cj1120c) lacking the N-terminal membrane domain, and containing an N-terminal His-tag. This version of PglF has been functionally characterized previously. Upon incubation of UDP-GlcNAc with PglF the expected product UDP-4-keto-6-deoxy-GlcNAc was formed.

As anticipated, incubation of UDP-4-keto-6-deoxy-GlcNAc with the mutant dehydratases oxidized the tightly bound cofactor to NADP\(^{+}\). The reaction of 50 μM D126N or K127A with 260 μM UDP-4-keto-6-deoxy-GlcNAc resulted in rapid disappearance of the 344 nm absorption band, such that upon returning the cuvette to the spectrometer (~15 s after the addition of UDP-4-keto-6-deoxy-GlcNAc) the NADPH absorption band had been quantitatively bleached. With Y135F the disappearance of the 344 nm band was dramatically slower. Upon incubation of 68 μM Y135F with 560 μM UDP-4-keto-6-deoxy-GlcNAc oxidation of the tightly bound NADPH was found to take 9 h to quantitatively bleach the NADPH absorption band at 25 °C.
The observed difference in rate of oxidation of the tightly bound NADPH in Y135F versus D126N or K127A is consistent with the anticipated role of tyrosine 135. Tyrosine 135 is part of the conserved triad found in SDR enzymes, and it is likely to be the catalytic residue involved in C-4" oxidation (B1, Figure 4.83, p. 134). The observation that NADPH bound to D126N or K127A, which both retain tyrosine 135, is so readily oxidized is consistent with this assignment as these mutants are clearly capable of promoting C-4" redox chemistry.

The complete oxidation of the cofactor in PseB with UDP-4-keto-6-deoxy-GlcNAc was a standard step during enzyme preparation prior to the experiments described below. Mutant and wild-type PseB with the catalytically relevant form of the nicotinamide cofactor bound were thus available, and a meaningful analysis of catalytic activities was possible.

4.3 Dehydratase Activities of PseB Wild-type, D126N, K127A and Y135F

The PseB catalyzed dehydration of UDP-GlcNAc in D2O was monitored by 1H NMR spectroscopy to determine if deuterium becomes incorporated at C-5" or C-6" of the UDP-arabino-sugar. The product 1H NMR spectrum has been assigned previously, and it is evident that spectral overlap of the H5" signal with other signals would make the H5" signal difficult to monitor directly. However, the H6" methyl signal of the UDP-arabino-sugar is well separated, allowing it to be monitored directly and unambiguously. To determine if deuterium becomes incorporated at C-6" the relative integration of the H6" signal was compared with that of the H1" signal. To determine if 1H or 2H is present at C-5" of product the multiplicity of the H6" signal was examined. The coupling constant of H5" to H6" is sufficiently large (J5',6" = 7.6 Hz) to be observed when 1H is at C-5", but only a singlet is anticipated to be observed when 2H is at C-5". Upon incubation of UDP-GlcNAc with PseB in D2O the H6" signals of hydrated and
unhydrated UDP-arabino-sugar were observed to grow in as singlets (Figure 4.89, Spectra A-C). The H5''/H6'' coupling, which is clearly obvious in a spectrum of UDP-arabino-sugar generated in H₂O (Figure 4.89, Spectrum D), is conspicuously lacking, indicating a deuterium has become incorporated at C-5'' of product (Figure 4.90). The H6'' product signal integrals are three times that of the H1'' product signal integrals, indicating that no deuterium was incorporated at C-6''. These observations clearly favour the proposed mechanism (Figure 4.83, p. 134) over the literature proposed mechanism (Figure 1.36, p. 39).

Figure 4.89 Partial ¹H NMR spectra during dehydration of 12.1 mM UDP-GlcNAc to UDP-arabino-sugar as catalyzed by 18 µM PseB at (400 MHz, D₂O, RT). A) UDP-GlcNAc in 10 mM potassium phosphate pH 7.0. B) Five minutes after the addition of PseB (9.7 % conversion). C) 90 min after the addition of PseB (74 % conversion). The small signal marked with an asterix (*) indicates the appearance of a new product after 90 min. D) Partial ¹H NMR spectrum of UDP-arabino-sugar prepared in H₂O showing strong H5''/H6'' coupling (J₅'',₆'' = 7.6 Hz).

The ratio of the unhydrated to hydrated product appears to vary as the PseB-catalyzed reaction proceeds (Figure 4.89). At 5 min the ratio is approximately one to one, while at 90 min the ratio approaches one to four, the observed equilibrium ratio for unhydrated to hydrated UDP-arabino-sugar. In incubations containing lower enzyme concentrations the unhydrated to
hydrated product ratio was observed to grow in at a one to four ratio. This is consistent with release of the unhydrated UDP-arabino-sugar from PseB, and hydration occurring slowly and non-enzymatically over the course of minutes (Figure 4.90).

Figure 4.90 PseB catalyzed dehydration of UDP-GlcNAc in D$_2$O produces UDP-[5-$^2$H]-arabino-sugar, which exists in the unhydrated and hydrated forms in aqueous solution. In addition, PseB catalyzes epimerization of the UDP-arabino-sugar to UDP-4-keto-6-deoxy-GlcNAc, which also exists in the unhydrated and hydrated forms in aqueous solution.

At high conversion new signals are observed in the $^1$H NMR spectrum (one signal can be seen in Figure 4.89, 90 min). These signals correspond to the unhydrated and hydrated signals of UDP-4-keto-6-deoxy-GlcNAc, the C-5" epimer of the UDP-arabino-sugar (Figure 4.90, lower section). The epimerization of the UDP-arabino-sugar to UDP-4-keto-6-deoxy-GlcNAc has been observed previously, however it was not clear if this epimerization is catalyzed by PseB or if it occurs non-enzymatically. This question was addressed in a second experiment, in which the reaction was taken to 86 % conversion, and PseB was removed via centrifugal filtration. The mixture was divided in two, and it was determined by $^1$H NMR spectroscopy that three sugar nucleotides were present: UDP-GlcNAc (14 %), UDP-arabino-sugar (82 %), and UDP-4-keto-6-deoxy-GlcNAc (4 %)(Figure 4.91). PseB was added to one sample while an equal volume of buffer was added to the control, and the mixtures were
monitored by $^1$H NMR spectroscopy. After 15 h 30 min no changes in the relative ratio of the three sugar nucleotides had occurred in the control sample, whereas in the PseB containing vessel UDP-4-keto-6-deoxy-GlcNAc was the only sugar nucleotide that could be detected. This experiment indicates that the C-5'' epimerization of UDP-\textit{arabino}-sugar to UDP-4-keto-6-deoxy-GlcNAc is PseB-catalyzed.

![Figure 4.91](image)

Figure 4.91 Partial $^1$H NMR spectra demonstrating the PseB-catalyzed nature of the epimerization of UDP-\textit{arabino}-sugar (○ = unhydrated, • = hydrated) to UDP-4-keto-6-deoxy-GlcNAc (Δ = unhydrated, ▲ = hydrated)(total UDP-sugar concentration = 4.1 mM, pH 7.0 potassium phosphate, D$_2$O, RT), in which spectra A-D) document the incubation with 91 μM PseB and spectrum, E) is the control with no enzyme. Spectra A-C) acquired at 300 MHz, spectra D, E) acquired at 400 MHz.

The C-5'' epimerase activity of PseB is ~100-fold less than the dehydratase activity. It is not likely to be physiologically relevant in \textit{C. jejuni}, as PglF appears to be a much better catalyst for the conversion of UDP-GlcNAc to UDP-4-keto-6-deoxy-GlcNAc. Regardless, the C-5'' epimerase activity complicates the study of PseB. Also, it is the reason why a completely
pure source of the UDP-arabino-sugar is not readily available, which will be relevant in the isotopic exchange experiments described in Section 4.4.

Specific activities for the dehydration of His-tagged wild-type and mutant PseB were measured with $^1$H NMR spectroscopy. Incubations were carried out in NMR spectroscopy tubes with 10 mM UDP-GlcNAc and 13 μM PseB in 12.5 mM potassium phosphate buffer (pD 7.4 D$_2$O). The initial 10 % of conversion to UDP-arabino-sugar was measured by comparing the integrals of the H6" signals of product to those of the combined N-acetyl signals. Under these conditions 5.1 % conversion was measured at 4.5 min with wild-type PseB, and the specific catalytic activity of His-tagged wild-type PseB was determined to be $0.23 \pm 0.05 \text{ μmol min}^{-1} \text{ mg}^{-1}$ at 23 °C. To measure the activities of the mutant dehydratases it was necessary to use 584 μM mutant enzymes in order to observe 5 % conversion within several days. The dehydratase activities of the mutants were found to be 0.001 to 0.002 % of the activity of wild-type PseB. It should be noted that UDP-4-keto-6-deoxy-GlcNAc was the observed product in the case of the mutants. This is consistent with the observation that the C-5" epimerase activity of the mutants was approximately equal to that of wild-type (discussed in more detail in Section 4.4).

During the above incubation of D126N, K127A, and Y135F with UDP-GlcNAc in D$_2$O, wash in of deuterium into substrate was considered. It was anticipated that deuterium could wash in to the H5" position via the reversible nature of the oxidation and dehydration steps (Figure 4.83, p. 134). As such the region of the $^1$H NMR spectrum containing the H5" signal was monitored. No significant changes were observed over three days, indicating that isotopic exchange into UDP-GlcNAc does not occur with the mutants.

The observation that these mutants all tightly bind the nicotinamide cofactor, yet are almost devoid of dehydratase activity is consistent with the proposal that they are catalytic residues. As NADPH bound to Y135F reacts extremely slowly with UDP-4-deoxy-6-deoxy-
GlcNAc, it appears that tyrosine 135 is required for C-4" redox chemistry. As this is the tyrosine of the SDR catalytic triad (Section 1.4, p. 5), the proposed role as catalytic base in C-4" redox chemistry is consistent with expectations based on analogy. NADPH bound to D126N or K127A was relatively easily oxidized by UDP-4-deoxy-6-deoxy-GlcNAc, suggesting that aspartate 126 and lysine 127 are not involved with C-4" redox chemistry. The impact of the mutation of these residues is devastating for dehydratase activity, suggesting that aspartate 126 and lysine 127 could be catalytic residues involved in the dehydration or reduction steps of the reaction. The following experiments were designed to provide evidence as to their exact roles in catalysis.

4.4 Solvent Isotopic Exchange into the C-5" position of the UDP-

arabino-sugar

In an effort to identify the catalytic residue that protonates C-5" of the α/β unsaturated ketone intermediate during the reduction step (B4, Figure 4.83, p. 134), the solvent isotope exchange into C-5" of the UDP-arabino-sugar product was investigated. To investigate the isotope exchange it was planned to incubate non-deuterated UDP-arabino-sugar with PseB in D₂O and monitor the wash in of deuterium at C-5" by observing the H6" signal. To this end the UDP-arabino-sugar was first prepared by executing the dehydration reaction of UDP-GlcNAc in H₂O. It was necessary to ensure that all the UDP-GlcNAc had been quantitatively consumed in this incubation, as any residual UDP-GlcNAc would generate UDP-[5-²H]-arabino-sugar upon incubation with PseB in D₂O (Figure 4.89). As PseB also epimerizes the UDP-arabino-sugar to form UDP-4-keto-6-deoxy-GlcNAc (Figure 4.91), it was not possible to generate a pure sample of the UDP-arabino-sugar by this method. The sample of UDP-arabino-sugar generated contained 56 % UDP-arabino-sugar and 44 % UDP-4-keto-6-deoxy-GlcNAc (Figure
Upon addition of wild-type PseB to this sample in D$_2$O the H6" doublet of the UDP-arabino-sugar is observed to diminish while an H6" singlet grows in, indicating that solvent deuterium washes into the C-5" position. The H6" singlet of the UDP-[5-$^2$H]-arabino-sugar is slightly shifted upfield, such that it overlaps with the upfield peak of the H6" doublet of the non-deuterated UDP-arabino-sugar. In an attempt to quantitate the deuterium wash-in the ratio of the peak heights was measured, and the fraction of deuterium washed in was calculated. In this way it was determined that deuterium wash-in at C-5" to the UDP-arabino-sugar occurs with a specific activity of 0.26 ± 0.08 μmol min$^{-1}$ mg$^{-1}$ at 23 °C with His-tagged wild-type PseB.

Figure 4.92  Partial $^1$H NMR spectra monitoring solvent isotopic exchange into the UDP-arabino-sugar catalyzed by 11.5 μM His-tagged wild-type PseB (○ and □ are downfield H6" signals of non deuterated sugars, ● and ■ are H6" signals of non-deuterated and overlapping ["H5"] sugars)(Total concentration of UDP-sugars = 10.5 mM, 56 % UDP-arabino-sugar and 44 % UDP-4-keto-6-deoxy-GlcNAc at t = 0, 10 mM pH 7.0 potassium phosphate, D$_2$O, 400 MHz).
The C-5″ epimerase activity of PseB was also observed on extended incubation (Figure 4.91, E). By integration of the H6″ signals it was determined that the amount of UDP-4-keto-6-deoxy-GlcNAc increased from 44 % to 57 % of the total amount of UDP-sugars after 19 h 30 min. Thus the specific activity of C-5″ epimerization of UDP-arabino-sugar for wild-type PseB was measured to be 0.002 ± 0.0015 μmol min⁻¹ mg⁻¹ under these conditions.

Upon extended incubation, a signal corresponding to H6″ of the UDP-[5-²H]-4-keto-6-deoxy-GlcNAc was observed to grow in (Figure 4.91, E). This singlet is slightly shifted upfield, such that that it overlaps with the upfield peak of H6″ doublet of the non-deuterated UDP-4-keto-6-deoxy-GlcNAc (similar to the analogous peaks of UDP-arabino-sugar). UDP-[5-²H]-4-keto-6-deoxy-GlcNAc can arise either from the C-5″ epimerase activity of PseB on the UDP-arabino-sugar or from washing deuterium directly into UDP-4-keto-6-deoxy-GlcNAc.

Incubation of D126N, K127A and Y135F with the same mixture of 56 % UDP-arabino-sugar and 44 % UDP-4-keto-6-deoxy-GlcNAc in D₂O allowed comparison of the mutant solvent isotope exchange and C-5″ epimerase activities with those of the wild-type. For the solvent exchange into H5″ of the UDP-arabino-sugar, the activities of all three mutants were ~100-fold lower than that of wild-type PseB (Table 4.3). With K127A and Y135F, the rate of isotopic exchange was greater than that of epimerization, although the activities are all small and the experimental error is large. Nevertheless, a comparison of these activities within a given mutant is meaningful, as both activities are occurring in the same vessel, under identical conditions. Thus, the 80 % faster rate of H5″ exchange versus epimerization for K127A, and the 40 % faster rate of H5″ exchange versus epimerization for Y135F are significant. Accordingly, the two activities are equal for D126N.
Specific Activity (μmol min$^{-1}$ mg$^{-1}$)  
<table>
<thead>
<tr>
<th>PseB</th>
<th>H5$^\prime$ Deuterium Exchange into UDP-arabino-sugar</th>
<th>C-5$^\prime$ Epimerization to UDP-4-keto-6-deoxy-GlcNAc</th>
<th>Activity Ratio$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.26 ± 0.08</td>
<td>0.002 ± 0.0015</td>
<td>130 ± 5</td>
</tr>
<tr>
<td>D126N</td>
<td>0.003 ± 0.0015</td>
<td>0.003 ± 0.0015</td>
<td>1.00 ± 0.09</td>
</tr>
<tr>
<td>K127A</td>
<td>0.006 ± 0.0015</td>
<td>0.003 ± 0.0015</td>
<td>1.78 ± 0.09</td>
</tr>
<tr>
<td>Y135F</td>
<td>0.004 ± 0.0015</td>
<td>0.003 ± 0.0015</td>
<td>1.41 ± 0.09</td>
</tr>
</tbody>
</table>

Table 4.3 Summary of the measured H5$^\prime$ solvent deuterium exchange into the UDP-arabino-sugar and C-5$^\prime$ epimerase activities of His-tagged wild-type, D126N, K127A and Y135F PseB measured by $^1$H NMR spectroscopy in 10 mM pH 7.0 potassium phosphate in D$_2$O at 23 °C. * H5$^\prime$ solvent deuterium exchange into UDP-arabino-sugar activity divided by C-5$^\prime$ epimerase activity.

The reason for this direct comparison of the two activities for each mutant is that the C-5$^\prime$ epimerase activity will also result in H5$^\prime$ exchange into UDP-arabino-sugar. Surprisingly, the C-5$^\prime$ epimerase activity does not require aspartate 126, lysine 127 and tyrosine 135, as the measured activities do not change for the three mutants studied. This suggests the C-5$^\prime$ epimerase activity does not occur in the same active site as dehydration. As the H5$^\prime$ exchange activity is equal to the epimerization activity for D126N, it is possible that the H5$^\prime$ exchange activity occurs solely due to epimerization in this second active site, and that the contribution from the dehydratase active site is negligible. This would implicate aspartate 126 as the catalytic residue that protonates C-5$^\prime$ during the reduction step of dehydration (B4, Figure 4.83, p. 134). However, the very low activities observed with all of the mutants prevent a definitive assignment. It is clear that each mutation has affected the ability of PseB to catalyze the H5$^\prime$ exchange reaction and the residues appear to act in concert.

4.5 Synthesis of UDP-6-deoxy-6-fluoro-GlcNAc

The strategy to identify the residue that protonates the C-6$^\prime$ hydroxyl during dehydration (B3, Figure 4.83, p. 134) required the synthesis of UDP-6-deoxy-6-fluoro-GlcNAc
23 (Figure 4.93). This synthesis is quite similar to those presented in Chapter Two, and as such it is only briefly discussed here. The strategy was to fluorinate the known compound 1,3,4-OAc-GlcNAc with DAST, as described previously. The known product, 1,3,4-OAc-6F-GlcNAc, was anomerically deacetylated and the phosphate was installed using a phosphoramidite reagent followed by mild oxidation. The known product, 24, was deprotected and treatment with UMP-morpholidate generated the known final product 23.

![Figure 4.93 Synthesis of UDP-6-deoxy-6-fluoro-GlcNAc 23.](image)

**Figure 4.93 Synthesis of UDP-6-deoxy-6-fluoro-GlcNAc 23.** a) TrCl, pyr., then Ac₂O. b) 50 psi H₂, Pd(OH)₂/C. c) DAST, CH₂Cl₂. d) Me₂NH. e) Et₂NP(OBn)₂, triazole. f) H₂O₂. g) H₂, Pd/C. h) NaOCH₃. i) UMP morpholidate, tetrazole.

### 4.6 Elimination of HF from UDP-6-deoxy-6-fluoro-GlcNAc 23

The incubation of 23 with PseB was monitored by ¹H and ¹⁹F NMR spectroscopy. After extended incubation a new signal at -122.8 ppm was observed in the ¹⁹F NMR spectrum (Figure 4.94). Addition of sodium fluoride enhanced this signal, indicating that fluoride is released from 23. In the ¹H NMR spectrum, signals corresponding to UDP-4-keto-6-deoxy-
GlcNAc were observed, indicating 15% conversion, as was observed by $^{19}$F NMR spectroscopy. No deuterium wash in at H5" of 23 was observed. No spectral changes were observed in a control sample of 23 in absence of PseB, indicating that the production of UDP-4-keto-6-deoxy-GlcNAc and fluoride are enzyme catalyzed. The observation of UDP-4-keto-6-deoxy-GlcNAc does not indicate that this is the first formed product, as after extensive incubation the C-5" epimerase activity of PseB would be expected to convert any UDP-arabino-sugar to UDP-4-keto-6-deoxy-GlcNAc. It is difficult to determine what the first formed product is from this experiment, as the UDP-arabino-sugar signals were not observed in $^1$H NMR spectra taken at earlier time points, indicating that the UDP-arabino-product does not accumulate during the incubation. It is also difficult to calculate an approximate activity for the PseB-catalyzed turnover of 23 from the spectra shown here, as PseB was found to precipitate at 37 °C. This does not affect the qualitative observation, however, that 23 is a poor substrate for PseB and that HF is released catalytically.

![Figure 4.94](image-url)

Figure 4.94 $^{19}$F NMR spectra during the incubation of 5 mM UDP-6-deoxy-6-fluoro-GlcNAc 23 with 25 μM PseB in 50 mM potassium phosphate pH 7.0 (282.4 MHz, D$_2$O). A) Spectrum immediately after addition of PseB (t = 0). B) After 18 h incubation at 37 °C. C) After addition of 2.4 μmol NaF to incubated sample.
The specific activity for the release of HF from 23 was ultimately measured for His-tagged wild-type PseB using a fluoride electrode. As PseB had been found to precipitate at 37 °C, these measurements were performed at 30 °C and no precipitation was observed. With 1 mM 23 and 43 μM PseB in 100 mM pH 7.0 potassium phosphate, a linear increase of fluoride concentration from 0 to 6 μM was observed over 100 min. Thus, the specific activity for release of HF from 23 was measured for His-tagged wild-type PseB to be 37 ± 9 pmol min\(^{-1}\) mg\(^{-1}\) at 30 °C. The elimination of HF (from 23) is four orders of magnitude slower than the normal dehydration reaction. When the concentration of 23 was increased to 2 mM the observed activity was 44 pmol min\(^{-1}\) mg\(^{-1}\), which is slightly higher than the previous value, but within the experiment error. This suggests that the substrate concentration is above \(K_m\) under these conditions, and that the \(k_{cat}\) for PseB-catalyzed elimination of HF from 23 is extremely low.

Despite the poor activity observed with wild-type, the fluoride electrode was used to screen the mutants for HF elimination activity. It was hoped that the mutant lacking the residue responsible for protonating the C-6" hydroxyl during the normal dehydration reaction (B3, Figure 4.83, p. 134) would display HF elimination activity equal to that of wild-type PseB, as the missing residue was anticipated to be nonessential for HF elimination. Unfortunately, such a scenario was not observed, as the fluoride electrode was unable to measure increases in fluoride concentration greater than the background noise (~0.2 μM) over 100 min (1 mM 23, 43 μM mutant PseB, 100 mM pH 7.0 potassium phosphate, 30 °C). Thus, only an upper limit was determined, and HF elimination activities of D126N, K127A, and Y135F are < 1 ± 0.9 pmol min\(^{-1}\) mg\(^{-1}\) at 30 °C, which is < 3 % of the wild-type activity. As none of the mutants displayed HF elimination activity of the same magnitude as wild-type activity, the anticipated result was not observed. No further effort was expended to measure the actual HF elimination activities of the mutants.
The observation that wild-type PseB is an extremely poor catalyst for the elimination of HF from 23 is not completely unexpected. An earlier experiment performed by Jackie Bassiri, a former M.Sc. student in the lab, investigated the reactivity of UDP-6-deoxy-6,6-difluoro-GlcNAc 25 with PseB. It had been proposed that 25 would be a suicide inhibitor of PseB as described in Figure 4.95. After the initial oxidation, HF would be eliminated and NADPH would deliver a hydride to C-6". The second fluoride would then be eliminated and the α,β unsaturated ketone thus generated could not be reduced. Instead, the α,β unsaturated ketone would react with an active site nucleophile, covalently modifying the active site. This strategy has been found to be effective with CDP-glucose 4,6-dehydratase (CGD), using the appropriate difluorinated substrate analog. Unfortunately, Ms. Jackie Bassiri found that incubation of 25 with PseB did not result in any observable changes to 25 or to PseB.

![Figure 4.95 Proposed suicide inactivation of PseB by UDP-6-deoxy-6,6-difluoro-GlcNAc 25. Ms. Jackie Bassiri found that incubation of 25 with PseB did not result in any observable changes to 25 or to PseB.](image)

Although it was not understood why 25 is unreactive with PseB, it seemed more likely that 23 would be reactive with PseB as it better resembles substrate. Indeed, incubation of 23
with PseB does result in HF elimination; however, this activity is $10^4$-fold less than that of the natural reaction. It had been hoped that PseB would exhibit HF elimination activity in line with dTDP-glucose 4,6-dehydratase (RmlB), which has a rate constant ($k_{cat}$) for HF elimination only 100-fold less than that of the natural reaction.\(^7\)

### 4.7 Summary and Future Research

In this study of PseB evidence was gathered that supports the proposed mechanism (Figure 4.83, p. 134) and is incompatible with the literature proposed mechanism (Figure 1.36, p. 39). The observation of solvent deuterium incorporation at H5" of the UDP-arabino-sugar is incompatible with delivery of the hydride to C-5" as proposed in the literature, but is entirely consistent with C-5" protonation during the reduction step and hydride delivery to C-6". This is in agreement with the three known non-inverting dehydratases discussed in Section 1.7 (p. 25).

The C-5" epimerization of the UDP-arabino-product has been investigated in this work, and the epimerase activity has been shown to be PseB-dependent. The epimerase activity is \(~100\)-fold lower than the natural dehydratase activity, and is independent of aspartate 126, lysine 127, or tyrosine 135, as mutation of these residues did not affect the observed epimerase activity. It is not clear which residues are involved.

The mutants D126N, K127A, and Y135F have been prepared and it has been shown that while these mutants are isolated with cofactor tightly bound, the cofactor is mostly in the wrong oxidation state for catalysis. It was found that UDP-4-keto-6-deoxy-GlcNAc is able to oxidize the tightly bound cofactor in situ, and that the relative rates for the cofactor reduction were dramatically different among the mutants. Under the conditions described, D126N and K127A both reacted instantaneously (within 15 s), demonstrating that these mutants are able to perform C-4" redox chemistry efficiently, though a thorough measurement of these rates or comparison
with wild-type was not pursued. The reaction of Y135F bound NADPH with UDP-4-keto-6-deoxy-GlcNAc was relatively slow. This is consistent with the proposal that tyrosine 135 is the catalytic base for C-4" redox chemistry. The in situ oxidation of the tightly bound cofactor allowed the measurement of several mutant activities that would otherwise be impossible to measure if the cofactor were in the reduced form.

The mutant dehydratases were found to lack any significant dehydratase activity, consistent with the proposal that they are catalytic residues. The measured activities are 50 000- to 100 000-fold lower than wild-type (Table 4.4). No wash in of solvent derived deuterium into UDP-GlcNAc was observed in these experiments.

Two alternate reactions of PseB were investigated. The first, solvent deuterium exchange at C-5" of the UDP-arabino-sugar was observed for wild-type PseB with a specific activity approximately equal to the dehydratase activity. The second reaction is the elimination of HF from UDP-6-deoxy-6-fluoro-GlcNAc 23, which occurs with an extremely low activity approximately $10^4$-fold slower than the natural dehydration. The extremely slow nature of the HF elimination is surprising from the perspective of an organic chemist, as fluoride is a reasonably good leaving group. Also, as fluorine is smaller than a hydroxyl one would not expect 23 to encounter steric problems upon binding to PseB. The hydrogen-bonding properties of fluorine are different than those of a hydroxyl group, however, and it could be that interruption of the hydrogen bonding network has devastating consequences on PseB elimination activity.

The activities of the mutants for solvent isotope exchange into product were measured and an upper limit for HF elimination activities was established, however this did not yield insight into the possible roles that these residues play (Table 4.4). The experiment investigating H5" deuterium exchange was hampered by the C-5" epimerase activity, which would also be
expected to wash deuterium into H5" of UDP-arabino-sugar. The wash in activity of D126N was identical to the C-5" epimerase activity, suggesting that the epimerization was solely responsible for isotope incorporation.

<table>
<thead>
<tr>
<th>PseB</th>
<th>Relative Activity (%)</th>
<th>Dehydration</th>
<th>Product H5&quot; Exchange</th>
<th>HF Elimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>D126N</td>
<td>0.002</td>
<td>1</td>
<td>&lt;3</td>
<td></td>
</tr>
<tr>
<td>K127A</td>
<td>0.0015</td>
<td>2</td>
<td>&lt;3</td>
<td></td>
</tr>
<tr>
<td>Y135F</td>
<td>0.001</td>
<td>1.5</td>
<td>&lt;3</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4 Summary of the relative activities of wild-type and mutant PseB for dehydration of UDP-GlcNAc (at 23 °C), H5" isotopic exchange into the UDP-arabino-sugar (at 23 °C), and elimination of HF from UDP-6-deoxy-6-fluoro-GlcNAc 23 (at 30 °C).

Failure to observe turnover of 23 with any of the mutants suggests that all three putative catalytic residues are essential for elimination. Again, it is possible that the hydrogen bonding network is finely tuned, and that any perturbation of hydrogen bonding is catastrophic for catalysis. This appears to be the major downfall of the strategies to identify catalytic residues described in this chapter.

In future work, many interesting questions concerning the catalytic activity of PseB need to be addressed. One promising experiment is to study the dehydration of both C-6" epimers of UDP-[4-2H,6-3H]-GlcNAc (Figure 4.96). It is anticipated that PseB will transfer the deuterium isotopic label from C-4" to C-6", generating a chiral methyl at C-6" of the product. As it appears from the crystal structure that the dehydration occurs via syn-elimination, as with all three known dehydratases (Section 1.7, p. 25), this experiment should reveal the stereochemistry of the reduction step. In addition, observation of intramolecular hydride transfer would confirm the proposed chemical mechanism.
4.8 **Final Comments on Mechanistic Investigation of HldD and PseB**

The mechanisms of two enzymes have been investigated in this thesis, and a contribution to their understanding has been made. While this is admittedly a small contribution to the enormous body of scientific knowledge, the contribution is novel and of interest in several respects. In the interest of human health, HldD and PseB are both linked to bacterial viability, and have no human counterpart. Thus, they are potential targets for drug development, and an understanding of their mechanisms is useful in the development of new drugs. The mechanisms of HldD and PseB are also unique among enzymes, and therefore interesting from a mechanistic standpoint. The understanding of these enzymes adds to the growing understanding of how enzymes are able to achieve catalysis. Finally, several strategies to elucidate the mechanisms of these enzymes have been tested, and it is instructive to an enzymologist to see which strategies were successful, and which strategies were not.

One particularly difficult aspect of enzymology is to determine with confidence the identities and roles of catalytic acid/base residues. In this thesis the general strategy was to
screen mutants for the ability to perform the natural reaction, and control reactions that probe only several steps of the natural reaction. It is interesting to observe that this strategy worked well for HldD, but was completely unsuccessful with PseB. HldD and PseB are similar enzymes of the SDR enzyme family and both utilize a tightly bound cofactor to catalyze chemistry via transient oxidation. However, the two enzymes catalyze completely different reactions. The relatively straightforward nonstereospecific chemistry catalyzed by HldD involves two redox steps, with each catalytic base operating independently of the other. This allowed a successful identification of the catalytic residues of HldD using the strategy described in this thesis. PseB, in contrast, catalyzes a much more complex reaction involving oxidation of an alcohol, elimination, and stereospecific reduction of an alkene, and was not amenable to this strategy to identify catalytic residues. This is not to say that complicated enzyme mechanisms cannot be tested. Rather, it should be noted that if the catalytic residues do not operate independently, then to identify catalytic acid/base residues and their roles, other strategies need to be explored. Clearly, the lack of one general, fool-proof strategy to elucidate enzymatic reactions and establish the identities and roles of catalytic acid/base residues underlines the continued need to develop new strategies to study enzyme mechanisms, and illustrates the unpredictable nature of enzyme mechanisms themselves.

4.9 Experimental

4.9.1 Materials and General Methods

All chemicals were purchased from Sigma-Aldrich and used without further refinement unless otherwise noted. Dry solvents were distilled fresh, using CaH₂ (CH₂Cl₂, pyridine) or Na/benzophenone (THF) as drying agent. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard.¹²⁶¹H-NMR spectra were obtained
on a Bruker AV300 or AV400 spectrometer at a field strength of 300 or 400 MHz, respectively. Proton-decoupled $^{31}$P NMR spectra were recorded on these spectrometers at 121.5 MHz or 162 MHz, respectively. Proton-decoupled $^{19}$F NMR spectra were obtained on the Bruker AV300 spectrometer at 282.3 MHz. Mass spectrometry was performed by electrospray ionization (ESIMS) using a Waters Micromass LCT mass spectrometer at the Mass Spectrometry/Microanalysis Laboratory at UBC. An Orion fluoride electrode (model 96-09BN), interfaced with a Fischer Scientific Accumet 925 pH/ion meter, was used to monitor fluoride release.

### 4.9.2 Site-Directed Mutagenesis to Prepare Wild-type and Mutant pseB

The plasmid pNRC20 was generously donated by collaborators at the National Research Council of Canada. The plasmid pNRC20 had been prepared by cloning the $pseB$ gene (also referred to as cj1293) from the *C. jejuni* subspecies *jejuni* (strain NCTC 11168) into a pET30 vector, which encodes for a C-terminal hexahistidine tag to the expressed protein ($PseB$ sequence-LEHHHHHHH). Unfortunately pNRC20 encodes two mutations to the nucleic acid sequence: g328t and t384c. The g328t mutation codes for a D110Y mutation in the protein sequence, and the t384c mutation is silent (does not change the expressed protein sequence). In the first round of mutagenesis the g328t mutation was corrected according to the protocol in the QuikChange Site-Directed Mutagenesis Kit from Stratagene, using Platinum Pfx DNA polymerase, $Dpn$ I restriction enzyme, and dNTPs from Invitrogen. The corrected plasmid encoding His-tagged wild-type $pseB$ was then used to prepare the mutant plasmids. Oligonucleotide primers used are listed below, with the mutated nucleotides underlined. The g328t mutation was corrected by mutagenesis using the following primers: $5'$-CGGTGCAGAAAATGTCATCGACGTTTGTTTTGAAAATGG-3' (forward), and: $5'$-CCATTTTCAAAACAAGCGTGACGATTTTTGCGCACC-3' (reverse). This created the
plasmid encoding the gene \textit{pseB} (t384c) which was used to express His-tagged wild-type PseB. Using the plasmid encoding the gene \textit{pseB} (t384c), the following primers were used to generate the plasmid encoding the gene \textit{pseB} D126N (t384c): 5'-TGTATCGCTTCTTAGTACGAATAAGGCCTGTAATCCTG-3' (forward), and 5'-CAGGATTACAGGCCCTTATTCGTACTAAGAGCGATACA-3' (reverse). The plasmid encoding the gene \textit{pseB} K127A was prepared using the following primers and the plasmid encoding the gene \textit{pseB} (t384c) as template. These primers encode for the K127A mutation and correct the silent mutation: 5'-GCTCTTAGTACGGATGCGGTTGTAATCCTG-3' (forward), and 5'-CAGGATTACAAAGCCGCATCCGTACTAAGAGC-3' (reverse). The plasmid encoding the gene \textit{pseB} Y135F (t384c) was prepared using the following primers and the plasmid encoding the gene \textit{pseB} (t384c) as template: 5'-GTAATCCTGTAAATTTATTCGGTGCAACCAAACTTGC-3' (forward), and 5'-GCAAGTTTGGTTGCACCGAATAAATTTACAGGATTAC-3' (reverse). Mutant gene sequences were confirmed by sequencing the entire gene.

4.9.3 Overexpression and Purification of Wild-type and Mutant PseB and P1gF

The plasmid pNRC40.1 that bears a truncated version of the \textit{pglF} (Cj1120c) gene from \textit{C. jejuni} subspecies \textit{jejuni} (strain NCTC 11168) was generously donated by collaborators at the National Research Council of Canada. The plasmid pNRC40.1 had been prepared using a pFO4 vector (a pET15b derivative in which the EcoRI-HindIII sites have been removed and replaced by the sequence encoding MGSSHHHHHHH) and encodes an N-terminal His-tagged version of PglF (residues 130-590).\textsuperscript{92}

Overexpression and purification of PseB and PglF was carried out as described for HldD and HldE (Section 2.5.3, p. 77). The only modification to the procedure was that \textit{E. coli}
BL21(DE3) cells were used for the overexpression of PseB and the purification by nickel affinity chromatography was carried out at 4 °C (PseB and PglF). Typical yields of purified protein were ~20 mg/L of cell culture. Both PseB and PglF could be stored frozen in the storage buffer (25 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 5 mM β-mercaptoethanol, and 10 % glycerol) at −80 °C for several months without loss in activity.

4.9.4 Preparation of UDP-4-keto-6-deoxy-GlcNAc with PglF in H₂O

UDP-GlcNAc (3 mg, 4.6 μmol) was dissolved in 400 μL 10 mM potassium phosphate buffer (pH 7.0), to which was added 50 μL of a 40 mg/mL solution of PglF (50 μmol), which had previously been exchanged into 10 mM potassium phosphate buffer (pH 7.0) by centrifugal filtration. After 12 h incubation at RT the enzyme was removed by centrifugal filtration and the filtrate was frozen and lyophilized to dryness. ¹H and ³¹P NMR spectroscopic analysis of the residue revealed that UDP-4-keto-6-deoxy-GlcNAc was the only sugar nucleotide present in the crude product, by comparison with the reported spectrum of UDP-4-keto-6-deoxy-GlcNAc. No further purification was of UDP-4-keto-6-deoxy-GlcNAc was performed. UDP-4-keto-6-deoxy-GlcNAc was stored frozen in solution at −20 °C for several weeks, or as a lyophilized powder for up to six months with no apparent decomposition.

4.9.5 In Situ Oxidation of the Tightly Bound NADPH Cofactor of PseB

In preparation for experiments involving the measurement of mutant activities, each mutant (~30 μM) was incubated with an excess of UDP-4-keto-6-deoxy-GlcNAc (250 μM, prepared as described in Section 4.9.4) in storage buffer (25 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 5 mM β-mercaptoethanol, and 10 % glycerol) for 12 h at RT. The sugar nucleotide solution was then removed by successive centrifugal filtration of affect a 1000-fold dilution.
4.9.6 Preparation of the UDP-arabino-sugar with PseB in H₂O

The UDP-arabino-sugar was found to partially decompose upon lyophilization from potassium phosphate buffered solutions, but not from pure distilled water. Thus the preparation of UDP-arabino-sugar was carried out in unbuffered water. PseB (3.8 µmol in 140 µL), which had been exchanged into distilled water by centrifugal filtration, was added to a 325 µL solution of UDP-GlcNAc (15.4 mg, 23.6 µmol) that had been neutralized with NaOH. The RT incubation was monitored by ¹H NMR spectroscopy, using an insert containing D₂O to allow for locking of the spectrometer. After 22 h all the UDP-GlcNAc had been consumed and the enzyme was removed by centrifugal filtration, and the solution was lyophilized to dryness. The mixture of UDP-arabino-sugar (56 %) and UDP-4-keto-6-deoxy-sugar (44 %) was stored as a dry solid at −80 °C for up to several weeks with no apparent decomposition.

4.9.7 Monitoring Enzyme Incubations by NMR Spectroscopy

The general procedure to monitor enzyme incubations by NMR spectroscopy is as follows. Following the in situ oxidation of the tightly bound NADPH as described in Section 4.9.5, PseB was exchanged into deuterated buffer (pD 7.4 potassium phosphate, D₂O) by successive centrifugal filtration of affect a 1000-fold dilution of the storage buffer. The enzyme concentration was measured using the Bradford assay. The enzyme solution was stored at 4 °C or on ice and used within a few hours. The substrate was dissolved with the same deuterated phosphate buffer and concentrations were measured by UV spectroscopy at 262 nm (molar absorptivity = 9890 M⁻¹ cm⁻¹ in 100 mM potassium phosphate, pH 7.0). Before the addition of enzyme, a spectrum of substrate alone was measured; this served as the zero time point. Incubations were initiated by addition of the enzyme to the NMR tube. Spectra were taken,
initially every 5 min, then after progressively longer time intervals. The specifics of each incubation experiment are described in the following sub-sections.

4.9.7.1 PseB-dependence of C-5’’ Epimerization

The PseB-dependence of C-5’’ epimerization of the UDP-arabino-sugar to UDP-4-keto-6-deoxy-GlcNAc was monitored as follows. A 1.00 mL solution of 5 mM UDP-GlcNAc, 32 μM PseB, and 10 mM potassium phosphate (pD 7.4, D2O) was incubated for 1 h, at which time the enzyme was removed by centrifugal filtration. Two 400 μL aliquots of the filtrate were placed into two different NMR tubes. By 1H NMR spectroscopy, it was determined that a mixture of 14 % UDP-GlcNAc, 82 % UDP-arabino-sugar, and 4 % UDP-4-keto-6-deoxy-GlcNAc (= UDP-sugar mixture) was present in the filtrate (Figure 4.91, Spectrum A, p. 144). PseB was added to one NMR tube to give 483 μL solution containing 91 μM PseB, 4.1 mM UDP-sugar mixture, and 10 mM potassium phosphate (pD 7.4). An 83 μL aliquot of 10 mM potassium phosphate (pD 7.4) buffer alone was added to the control. Both tubes were incubated at 23 °C.

4.9.7.2 Monitoring Dehydratase Activity

Dehydratase activities were monitored with 10 mM UDP-GlcNAc in 12.5 mM potassium phosphate (pD 7.4) at 23 °C. The solution volume of WT and mutant incubations were all 400 μL, although the concentration of enzyme was 13 μM in the case of WT PseB, and 584 μM in the case of D126N, K127A, and Y135F.

4.9.7.3 Monitoring Solvent Isotopic Exchange Activity

Solvent isotope exchange into the C-5’’ position of the UDP-arabino-sugar product activities for WT and mutants were monitored using the sample of UDP-arabino-sugar
prepared as described in Section 4.9.6, which contained 56% of the UDP-arabino-sugar and 44% of UDP-4-keto-6-deoxy-GlcNAc (= UDP-sugar mixture). Each NMR tube contained a 450 μL solution of 10.5 mM of the UDP-sugar mixture, 10 mM potassium phosphate (pD 7.4), and 11.5 μM of either WT, D126N, K127A, and Y135F. The temperature during this experiment was 23 °C. The C-5” epimerase activities were also monitored in this experiment.

4.9.7.4 HF Elimination from 23

The PseB-catalyzed elimination of HF from 23 was monitored in a 400 μL solution containing 5 mM 23, 25 μM WT PseB and 50 mM potassium phosphate (pD 7.4) at 23 °C. A control sample of 23 under identical conditions, only lacking PseB, was also monitored. No spectral changes were observed in either tube after 30 min, therefore the incubation temperature was increased to 37 °C. After 18 h at 37 °C, precipitate was observed in the PseB-containing sample.

4.9.8 Synthesis of Uridine 5’-(2-acetamido-2,6-dideoxy-6-fluoro-α-D-glucopyranosyl) Diphosphate, Disodium Salt 23

A 3:1 α/β anomeric ratio of 2-acetamido-1,3,4-tri-O-acetyl-6-trityl-2-deoxy-D-glucopyranose was prepared as described previously, and purified by column chromatography (2:1 ethyl acetate/petroleum ether to 100 % ethyl acetate, Rf = 0.4 in ethyl acetate). The trityl group was removed by hydrogenolysis to generate 1,3,4-tri-O-acetyl-2-deoxy-D-glucopyranose as described here. To a solution of 2.33 g of 2-acetamido-1,3,4-tri-O-acetyl-6-trityl-2-deoxy-D-glucopyranose (3.95 mmol) in 100 mL methanol 1.60 g 20 % palladium hydroxide on carbon was carefully added. The mixture was shaken in a Parr hydrogenator under 50 psi of hydrogen for four days. After filtration through celite the filtrate was dried in vacuo. Column chromatography (19:1 ethyl acetate to methanol) allowed isolation
of fractions with Rf = 0.3, which upon removal of solvent in vacuo proved to be 1.17 g (3.36 mmol, 85 % yield) of the known compound 1,3,4-tri-O-acetyl-2-deoxy-D-glucopyranose, as verified by 1H NMR spectroscopy.\textsuperscript{141} Introduction of the fluorine at C-6 was achieved by treatment of 1,3,4-tri-O-acetyl-2-deoxy-D-glucopyranose with DAST, which has been described previously.\textsuperscript{142} Purification by column chromatography (1:1 CH₂Cl₂/ethyl acetate to 100 % ethyl acetate) gave the known compound 2-acetamido-1,3,4-tri-O-acetyl-2,6-dideoxy-6-fluoro-D-glucopyranose, as identified by 1H and 19F NMR spectroscopy.\textsuperscript{143} The anomeric position of 2-acetamido-1,3,4-tri-O-acetyl-2,6-dideoxy-6-fluoro-D-glucopyranose (456 mg, 1.31 mmol) was then selectively deacetylated using dimethylamine, as described in Section 2.5.4.1 (p. 79). The crude mixture was separated by column chromatography (3:1 ethyl acetate/petroleum ether to 100 % ethyl acetate) and the fraction with Rf = 0.25 (ethyl acetate) was collected, and the 1H NMR spectrum was consistent with the isolation of 180 mg 2-acetamido-3,4-di-O-acetyl-2,6-dideoxy-6-fluoro-D-glucopyranose (0.59 mmol, 45 % yield). 2-acetamido-3,4-di-O-acetyl-2,6-dideoxy-6-fluoro-D-glucopyranose (417 mg, 1.36 mmol) was then phosphorylated using the standard procedure described in Section 2.5.4.2 (p. 79). Purification by column chromatography (9:1 diethyl ether/ethyl acetate to 100 % ethyl acetate) yielded 150 mg (0.26 mmol, 20 % yield) of the known compound 2-acetamido-3,4-di-O-acetyl-2,6-dideoxy-6-fluoro-α-D-glucopyranosyl dibenzyl phosphate \textsuperscript{24} as identified by 1H and 19F NMR spectroscopy (Rf = 0.35 in ethyl acetate).\textsuperscript{144} The benzyl protecting groups were then removed from compound \textsuperscript{24} (50 mg, 88 μmol) using the standard hydrogenolysis procedure described in Section 2.5.4.3 (p. 80). The crude product was then deacetylated with sodium methoxide by stirring in 3 mL of 100 mM sodium methoxide in methanol for 1 h. The reaction solution was neutralized by addition of Amberlite IR-120H resin (proton form). Removal of the acetyl protecting groups was confirmed by 1H NMR spectroscopy. To prepare for the morpholidate coupling reaction
the deprotected sugar was maintained under vacuum for 24 h. One equivalent of trioctylamine (88 μmol, 39 μL) was then added with 2 mL distilled pyridine, and the solvent was removed in vacuo. An additional 2 mL distilled pyridine was added then removed in vacuo. This was repeated twice more, and again after the addition of 20 mg of 1H-tetrazole (0.28 mmol) and 91 mg UMP morpholidate (0.13 mmol). Finally 0.75 mL distilled pyridine was added and the solution was stirred for 3 days under argon at RT. The pyridine was then removed in vacuo and the residue was dissolved in 10 mL water, and then washed with 2 x 10 mL diethyl ether. The aqueous layer was then separated by anion exchange chromatography (DE-52 resin, linear gradient from 0.1 M to 0.5 M of triethylammonium bicarbonate buffer, pH 7.5). Fractions containing the 23 were flash frozen and lyophilized, pooled, and passed through a column of Amberlite IR-120(+) resin (sodium form). Pooled fractions were dried in vacuo, and the known compound uridine 5′-(2-acetamido-2,6-dideoxy-6-fluoro-α-D-glucopyranosyl) diphosphate, disodium salt 23 was identified and characterized by NMR spectroscopy and mass spectrometry, which is fully summarized here. UV measurement (molar absorptivity = 9890 M⁻¹ cm⁻¹ at 262 nm, 100 mM potassium phosphate, pH 7.0) determined that 20 μmol of 23 was isolated (23 % yield). ¹H NMR (D₂O): δ 7.93 (d, 1H, J₅₆ = 7.9 Hz, H₆), 5.92-5.98 (m, 2H, H₁′, H₅), 5.51 (dd, 1H, J₁1''p = 6.98 Hz, J₁1''2'' = 3.05 Hz, H₁''), 4.59-4.85 (obscured by solvent peak, H₆''a and H₆''b), 4.31-4.37 (m, 2H, H₅'a, H₅'b), 4.13-4.28 (m, 3H, H₂', H₃', H₄'), 4.03 (dd, 1H, J₅''₁'' = 30.5 Hz, J₄''₅'' = 9.6 Hz, H₅''), 3.99 (d, 1H, J₂''₃'' = 10.5 Hz, H₂''), 3.81 (dd, 1H, J₂''₃'' = J₃''₄'' = 9.6 Hz, H₃''), 3.63 (dd, 1H, J₃''₄'' = J₄''₅'' = 9.8 Hz, H₄''), 2.05 (s, 3H, COCH₃); ³¹P NMR (D₂O): δ −8.96 (d, 1P, J₁₁P = 21.7 Hz, P₁), −10.83 (d, 1P, J₁₁P = 21.8 Hz, Pβ); ¹⁹F NMR (D₂O): δ −236.88 (s); ESIMS: m/z 608.1 [M − 2 Na + H]⁻.
4.9.9 Measuring Release of Fluoride from 23 using a Fluoride Electrode

Following the in situ oxidation of the tightly bound NADPH as described in Section 4.9.5, wild-type and mutant dehydratases were exchanged into 100 mM potassium phosphate buffer by centrifugal filtration. Enzyme solutions were diluted appropriately to allow for a 300 μL solution with 44 μM of enzyme and incubated for 15 min at 30 °C with the electrode inserted to allow for equilibration. Reactions were initiated by adding 6.0 μL of a 50 mM solution of 23 (generating a 1.0 mM solution of 23) and the incubation was followed for 100 min.
REFERENCES


Figure A.97  $^1$H NMR spectrum of adenosine diphosphate 6-deoxy-6-fluoro-β-D-mannose 1, bis(triethylamine) salt (D$_2$O, 300 MHz).
Figure A.98

$^1$H NMR spectrum of adenosine $\beta$-D-manno-hexofaldose diphosphate 8.

bis(triethylamine) salt (D$_2$O, 400 MHz).
Figure A.99: NMR spectrum of adenosine 13-D-mannose diphosphate 9, bis(trimethylamine) salt (D2O, 400 MHz).
Figure A.100 NMR spectrum of adenosine P-D-mannuronate diphosphate 10, bis(triethylamine) salt (D$_2$O, 400 MHz).
Figure A.101  $^1$H NMR spectrum of mannose dialdehyde 20 (D$_2$O, 400 MHz).
Figure A.102. $^1$H NMR spectrum of adenosine 6-azido-6-deoxy-β-D-mannose diphosphate 22, bis(triethylamine) salt (D$_2$O, 400 MHz).