AN INVESTIGATION OF THE MECHANISM OF UDP-GLUCOSE DEHYDROGENASE

Lisa C. Penney
B.Sc. (Honours), Memorial University of Newfoundland, 1999

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE STUDIES
Department of Chemistry

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
December, 2001

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Chemistry

The University of British Columbia
Vancouver, Canada

Date Jan. 23/02.

ABSTRACT

UDP-glucose dehydrogenase (UDPGluDH) is an NAD^+-dependent enzyme that catalyzes the two-fold oxidation of UDP-glucose to UDP-glucuronic acid. This enzyme is interesting in that its single active site carries out two sequential oxidations, whereas the majority of NAD^+-dependent dehydrogenases catalyze only a single oxidation step. Bacterial UDPGluDH is necessary for the formation of an antiphagocytic capsule that protects many virulent strains from the host's immune system. It has been determined that the enzymatic mechanism proceeds through covalent catalysis using an active site cysteine residue. Hydrolysis of the resulting thioester intermediate generates the UDP-glucuronic acid product.

The structure of wild-type UDPGluDH has been previously elucidated in our laboratory. This allowed residues that may be important in the enzyme's catalytic mechanism to be identified and several mutant forms of the enzyme to be constructed. Three of these mutants – Thr118Ala, Glu141Gln, and Glu145Gln – have been purified to homogeneity and their K_m and k_{cat} values have been determined. It was found that the k_{cat} value of the Thr118Ala UDPGluDH was reduced by a factor of 100 when compared to that of the wild-type enzyme. When this mutant enzyme was incubated with UDP-(6,6-di-^{2}H)glucose, the value of k_H/k_D was determined to be 1.9 ± 0.1, indicating that a C-H bond is broken in the enzyme's rate-determining step. This differs from previously obtained results using the wild-type enzyme, in which it was found that the hydride transfer steps were not rate-limiting. Thus, it appears that Thr118 plays a more important role in one or both of the hydride transfer steps of the enzymatic mechanism than in the hydrolysis of the thioester intermediate.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND SYMBOLS

6PGDH	6-phosphogluconate dehydrogenase
Å	angstrom (10^{-10} meter)
δ	chemical shift
ε	extinction coefficient (M^{-1})
h	Planck's constant
μM	micromolar (10^{-6} mole $liter^{-1}$)
ν	frequency of a stretching vibration
A_{600}	absorbance at 600 nm
ATP	adenosine triphosphate
BSA	bovine serum albumin
cap3a	the gene that encodes for UDPGluDH in *S. pneumoniae*
Cys260Ser	UDPGluDH with cysteine 260 replaced by a serine
D	deuterium
D264N	UDPGluDH with aspartate 264 replaced by an asparagine
Da	dalton (the mass of a hydrogen atom)
DE52	diethylaminoethylcellulose ion exchange resin
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E141Q	UDPGluDH with glutamate 141 replaced by a glutamine
E145Q	UDPGluDH with glutamate 145 replaced by a glutamine

E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetate (sodium salt)
ESI MS	electrospray ionization mass spectrometry
GDPManDH	guanosine-5'-diphosphomannose dehydrogenase
hasB	the gene that encodes for UDPGluDH in *S. pyogenes*
IPTG	isopropyl-1-thio-β-D-galactopyranoside
K204A	UDPGluDH with lysine 204 replaced by an alanine
k_{cat}	turnover (s^{-1})
kDa	kilodalton (10^3 dalton)
kfaC	the gene that encodes for UDPGluDH in *E. coli* K5
K_m	Michaelis constant
LB	Luria Bertani medium
LSIMS	liquid secondary ionization mass spectrometry
M	molar (mole $liter^{-1}$)
mM	millimolar (10^{-3} molar)
N208A	UDPGluDH with asparagine 208 replaced by an alanine
NAD(H)	oxidized or reduced nicotinamide adenine dinucleotide
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
PMSF	phenylmethylsulfonyl fluoride

ppm	parts per million
psi	pounds per square inch
rpm	revolutions per minute
S. pneumoniae	*Streptococcus pneumoniae*
S. pyogenes	*Streptococcus pyogenes*
SCHAD	L-3-hydroxyacyl-CoA dehydrogenase
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S_N2	second order nucleophilic substitution
T118A	UDPGluDH with threonine 118 replaced by an alanine
Trien	triethanolamine ($N(C_2H_4OH)_3$)
UDC	uridine 5'-diphosphate chloroacetol
UDP	uridine 5'-diphosphate
UDPG	UDP- glucose
UDPGA	UDP-glucuronic acid
UDPGluDH	UDP-glucose dehydrogenase
UDPManNAcDH	UDP-*N*-acetylmannosamine dehydrogenase
UTP	uridine 5'-triphosphate
UV	ultraviolet
Vis	visible
V_{max}	maximal reaction rate

Standard Abbreviations for Amino Acids

A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartate
E	Glu	glutamate
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
Y	Tyr	tyrosine

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to my supervisor, Dr. Martin Tanner, for giving me the opportunity to work in his research laboratory over the past two years. It has taught me a lot and I appreciate all of his assistance and guidance. I would also like to thank the past and present members of the Tanner group for their help in the completion of this project. They definitely made the lab an interesting place in which to spend many hours each day.

On a more personal note, I would like to thank my parents, Mary and Joe, for their support and encouragement over the past two years. It has been a rocky road at times and I don't know how far along I would have come without their love. I would also like to thank my friends, both long-time and recent, for their role in my personal development since coming to Vancouver. I've become a better person with their assistance, and that has taught me more than anything that I've ever learned in books has.

"Thanks, b'y..."

This thesis is dedicated to Figuring Things Out.

CHAPTER 1:

INTRODUCTION

UDP-glucose dehydrogenase (UDPGluDH) catalyzes the NAD^+-dependent, two-fold oxidation of UDP-glucose (UDPG) to UDP-glucuronic acid (UDPGA).

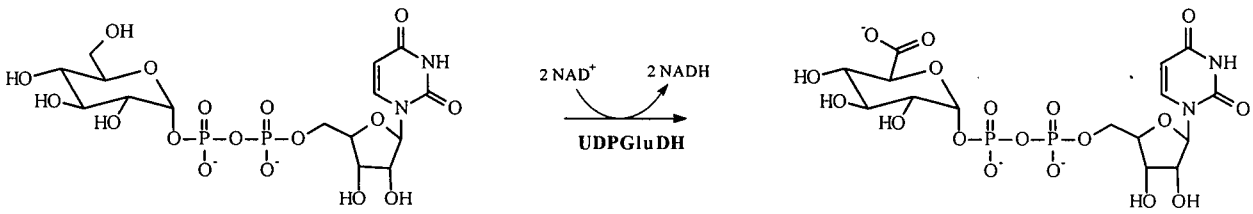


Figure 1.1 Reaction catalyzed by UDPGluDH.

The majority of NAD^+-dependent dehydrogenases catalyze a single oxidation. For example, an alcohol dehydrogenase converts an alcohol to an aldehyde, and an aldehyde dehydrogenase oxidizes an aldehyde to a carboxylic acid. Thus, two such enzymes would normally be required to perform the overall transformation catalyzed by UDPGluDH. There are only a few known NAD^+-dependent dehydrogenases that are capable of performing a two-fold oxidation within a single active site. UDPGluDH, along with its homologues UDP-*N*-acetylmannosamine dehydrogenase (UDPManNAcDH) and GDP-mannose dehydrogenase (GDPManDH), is one of these (1). The defining feature of this class of enzymes is their ability to perform a two-fold oxidation without the release of an aldehyde intermediate.

This introductory chapter will summarize previous research on UDPGluDH – from the initial discovery of the bovine liver enzyme in 1954 to the recent X-ray structure of the enzyme from *Streptococcus pyogenes*.

1.1 UDP-GLUCOSE DEHYDROGENASE

1.1.1 Biological Roles of UDPGluDH

UDP-glucose dehydrogenase is an important enzyme in both eukaryotes and prokaryotes and provides the only pathway for the formation of UDP-glucuronic acid in all organisms except plants. UDPGA is the activated donor of the glucuronic acid moiety that plays critical roles in a variety of organisms. In mammals, UDPGA is essential for the biosynthesis of various glycosaminoglycans such as heparin sulfate and hyaluronic acid. These polysaccharides are important components of connective tissues such as cartilage and blood vessel walls. UDPGA also plays a role in the secretion of waste metabolites from the liver. It forms glycosidic links to various nonpolar poisons and degradation products, causing them to become soluble in aqueous media. They can then be excreted from the body in the form of urine or bile (2). In plants, UDPGluDH may be an important regulatory enzyme in the carbon flux towards cell wall and glycoprotein biosynthesis due to feedback inhibition from UDP-xylose (3).

In many strains of pathogenic bacteria, such as group A streptococci (4) and *Streptococcus pneumoniae* type 3, UDPGA is required for the construction of a polysaccaride antiphagocytic capsule (5). The proper formation of this capsule is essential for bacterial virulence, as it serves to protect the bacteria from the host's immune system (6,7). This makes UDPGluDH a logical target for the development of pharmaceutical agents to combat these virulent organisms.

1.1.2 Studies on Bovine Liver UDPGluDH

UDPGluDH was first detected in bovine liver in 1954 (8) and was purified to homogeneity fifteen years later (9). The majority of the previous work towards elucidation of the enzyme mechanism was done by Kirkwood during the 1970's (10-14). This research culminated in the mechanism proposed in Figure 1.2.

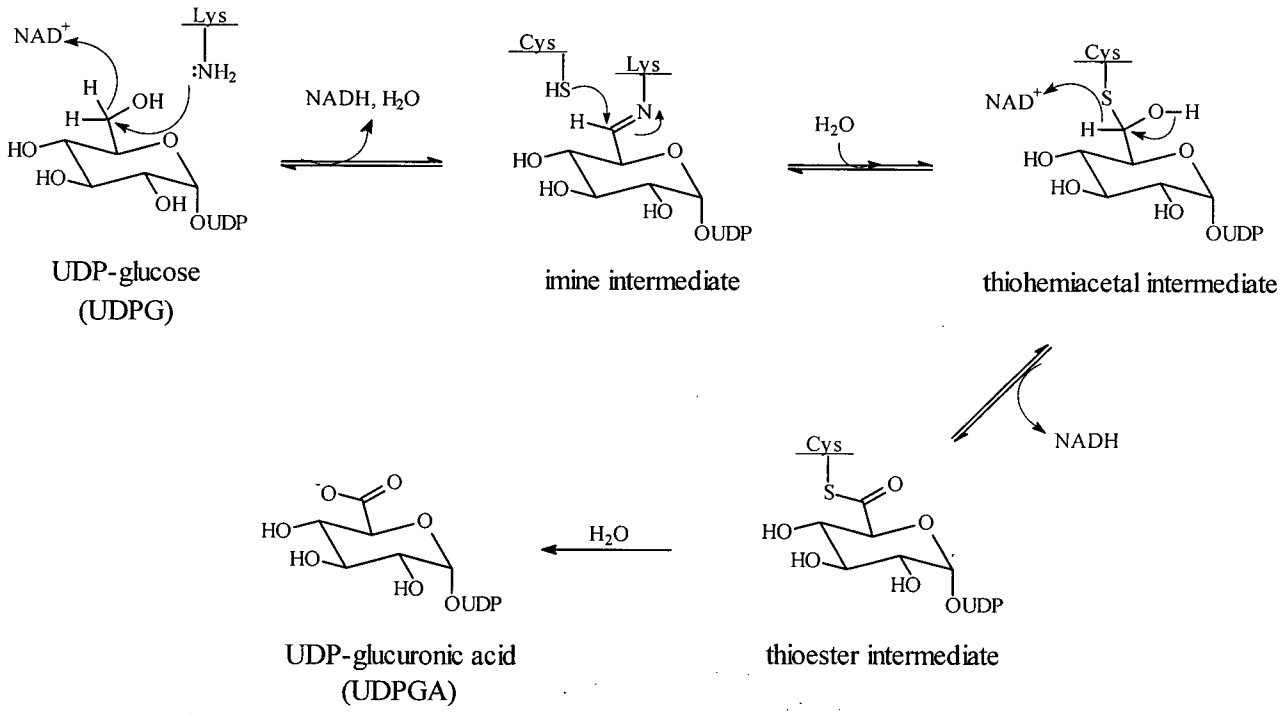


Figure 1.2 Kirkwood's proposed mechanism for UDPGluDH.

The first oxidation proceeds with the chemically unprecedented S_N2 displacement of a hydride to give a covalently bound imine intermediate. The second oxidation involves the unusual displacement of an amine by water, in which the enzyme bound imine is transferred to an active site cysteine to form a thiohemiacetal intermediate. A hydride transfer from the thiohemiacetal takes place to produce an enzyme bound thioester intermediate. A final irreversible hydrolysis of the thioester produces the product, UDPGA.

This mechanism remained unchallenged in the literature for almost 20 years, despite its apparent flaws. It accounts for the fact that an aldehyde intermediate in the enzymatic reaction had never been detected; it simply was not a true intermediate. The main line of evidence that led to this conclusion came from experiments with UDPGluDH in which the essential active site cysteine residue was covalently derivatized as a thiocyanate (13). Incubation of the thiocyanate-modified enzyme with UDPG and NAD^+, followed by reduction with $NaBH_4$, generated a covalent enzyme adduct. It was identified as a reduction product of the Schiff's base formed between the aldehyde and an active site lysine residue. This led to the proposal that the imine was a true intermediate in the enzymatic reaction.

One inconsistency with Kirkwood's mechanism comes from earlier studies on solvent oxygen isotope incorporation (15). When the reaction was carried out in ^{18}O labelled water, only one solvent oxygen atom was incorporated into the carboxylate of the final product. One might expect that formation of an imine intermediate would result in both oxygen atoms deriving from the solvent. To be consistent with the proposed mechanism, a sequestered water molecule from the original alcohol would have to be delivered back during the hydrolysis of either the imine or thioester intermediates.

1.2 CHARACTERISTICS OF BACTERIAL UDPGluDH

The first reports of the purification and characterization of bacterial UDPGluDH from *Escherichia coli* were published in the 1970's (16, 17). The enzyme is reported to be a homodimer with a subunit molecular weight of 47 kDa. It contains 2 cysteine residues per subunit and has a pH optimum of 9.0. A reducing agent such as 2-mercaptoethanol is necessary to maintain maximum activity. The K_m values are 1.0 mM for UDPG and 0.05 mM for NAD^+.

The first UDPGluDH gene to be cloned and sequenced was *hasB* from *S. pyogenes*, an encapsulated group A streptococcal strain (4). UDPGluDH from *S. pneumoniae* has since been cloned and overexpressed in *E. coli* (18), as has the *kfaC* gene of *E. coli* K5 (2). There is 74 % sequence similarity and 57% sequence identity between the enzymes from *S. pyogenes* and *S. pneumoniae* (5, 19).

Through a collaboration with Ivo van de Rijn at Wake Forest University, the recombinant protein from *S. pyogenes* was made available to us in a suitable expression vector. This enzyme has since been the subject of thorough investigation in our laboratory and a summary of the characterization results is presented in this section (20). It is a polypeptide chain consisting of 402 amino acids, with an expected molecular weight of 45 487 Da. The enzyme is sensitive to oxidizing conditions and rapidly loses activity in the absence of the reducing agent dithiothreitol (DTT), which must be included in the purification, storage, and assay buffers. This sensitivity is probably due to the rapid oxidation of a critical cysteine thiol that is important in the catalytic mechanism. It was also determined that UDPGluDH is much more stable at 5 °C than at 30 °C, and glycerol is required to protect the enzyme during repeated freeze-thaw cycles.

The optimum pH for activity in *S. pyogenes* UDPGluDH was determined to be 8.5 – 9.0. The enzymatic reaction was not inhibited by EDTA, suggesting that a divalent metal ion is not involved in catalysis. The overall reaction was not affected by carbonyl trapping reagents, indicating that if an aldehyde intermediate is formed during the course of the enzymatic reaction, it is sequestered from the bulk solvent. There was no evidence that UDPGluDH contains a tightly bound NAD^+ cofactor. The results from gel-filtration chromatography studies initially indicated that the enzyme exists as a monomer in solution. Subsequent X-ray structural studies have revealed that *S. pyogenes* UDPGluDH is actually a dimer in its active form (21). This

discrepancy may be indicative of a monomer/dimer equilibrium that depends on the presence of substrate or inhibitors.

1.3 THE KINETIC MECHANISM OF UDPGluDH

A chemically reasonable mechanism for UDPGluDH is outlined in Figure 1.3. In this proposed mechanism, the initial NAD^+-dependent oxidation of UDPG produces a tightly bound aldehyde intermediate. An active site cysteine residue then attacks the aldehyde, producing a thiohemiacetal intermediate. Collapse of the thiohemiacetal, along with a second hydride transfer to NAD^+, produces a thioester intermediate that is covalently attached to the active site cysteine. Hydrolysis of the thioester produces the product, UDPGA.

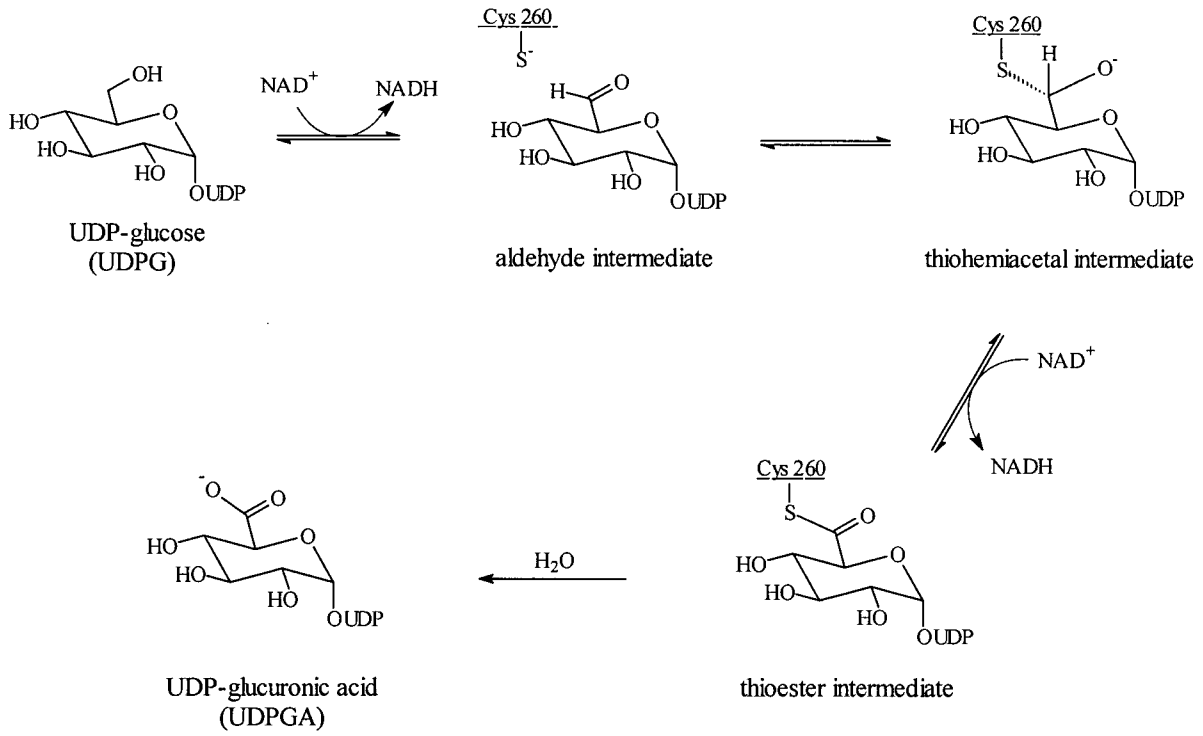


Figure 1.3 The new proposed mechanism of UDPGluDH.

The major differences between the mechanism proposed in Figure 1.3 and that proposed by Kirkwood is that an aldehyde intermediate is formed and there is no imine intermediate involved. Recent research in our laboratory has provided overwhelming support for this mechanism and many of the fine details have been determined. These will be outlined in the following sections.

1.3.1 Order of Reactant and Product Binding and Release

The initial velocity and product inhibition kinetic studies indicate that UDPGluDH follows a Bi Uni Uni Bi ping pong mechanism (20), with UDPG bound first and UDPGA released last. One molecule of NADH is released before the second NAD^+ is bound. This is shown schematically in Figure 1.4.

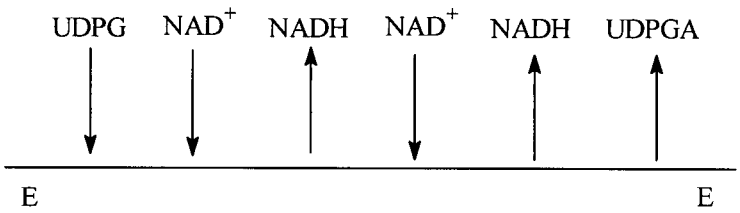


Figure 1.4 Proposed Bi Uni Uni Bi ping pong kinetic mechanism of UDPGluDH.

This mechanism has also been proposed for UDPGluDH from bovine liver (22).

1.3.2 Order of Hydride Transfer

As indicated in Figure 1.5, the two C6" hydrogen atoms of UDP-glucose can be designated as either pro-R or pro-S.

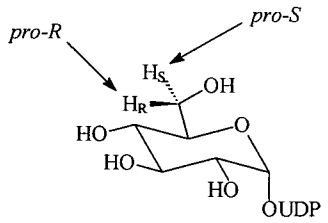
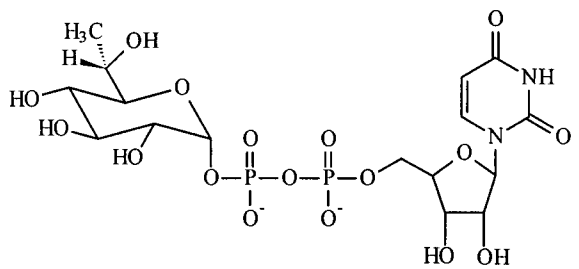
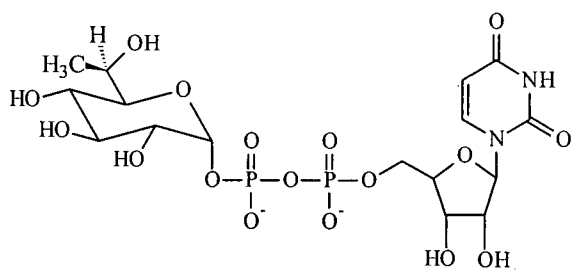


Figure 1.5 The prochiral hydrogen atoms of UDP-glucose.

The first oxidation of UDPG to the aldehyde intermediate involves a transfer of one of these hydrogens to NAD^+. To determine which hydrogen is initially removed, two secondary alcohol analogues of UDPG, uridine 5'-(7-deoxy-L-*glycero*-α-D-*gluco*-heptopyranosyl diphosphate) (**1a**) and uridine 5'-(7-deoxy-D-*glycero*-α-D-*gluco*-heptopyranosyl) (**1b**) (Figure 1.6), were synthesized (23). The oxidation of these molecules would produce a ketone product that would be unable to undergo further reaction. This single oxidation would be analogous to the normal alcohol to aldehyde oxidation. Thus, it would be expected that only one of the two diastereomers would be a substrate for UDPGluDH since the first hydride transfer would be dictated by the stereospecificity of the enzyme.



1a



1b

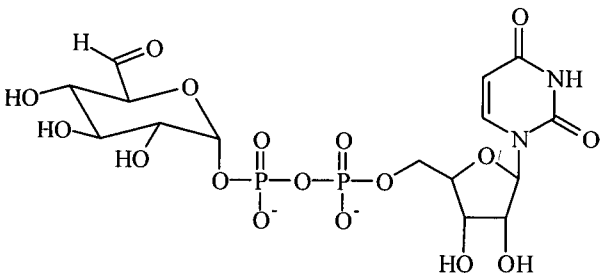
Figure 1.6 Uridine 5'-(7-deoxy-L-*glycero*-α-D-*gluco*-heptopyranosyl diphosphate) (**1a**), and uridine 5'-(7-deoxy-D-*glycero*-α-D-*gluco*-heptopyranosyl diphosphate) (**1b**).

It was determined that only **1a**, the analogue that retains the '*pro-R-like*' hydrogen of UDPG, is a substrate for UDPGluDH (23). This suggests that during the normal enzyme-catalyzed oxidation of UDPG to the aldehyde intermediate, the *pro-R* hydride is transferred to the first molecule of NAD^+. The remaining *pro-S* hydride must be transferred during the second oxidation of the thiohemiacetal to the thioester intermediate. These results agree with a previous report that bovine liver UDPGluDH transfers the *pro-R* hydride in the first oxidation step (11).

1.3.3 Evidence for the Formation of an Aldehyde Intermediate

The reaction catalyzed by UDPGluDH from *S. pyogenes* was carried out in ^{18}O-labelled water. A MALDI TOF mass spectrometric analysis of the UDPGA produced from this reaction indicated that a single solvent oxygen atom had been incorporated into the final product. The results from a ^{13}C NMR spectrum indicated that the label resided in the carboxyl group (24). Previous research on bovine liver UDPGluDH produced a similar result (refer to Section 1.1.2 and ref 15). This suggests that an imine intermediate is unlikely to participate in the reaction pathway and provides strong support for the formation of a tightly bound aldehyde intermediate.

The assumed aldehyde intermediate, uridine 5'-(α-D-*gluco*-hexodialdo-1,5-pyranosyl diphosphate) (**2**) (Figure 1.7), was chemically synthesized and tested as a substrate in the UDPGluDH reaction (25). It was found that the kinetic constants for the oxidation of **2** (k_{cat} = 1.0 s^{-1} and K_m = 14 μM) were very similar to those obtained for UDPG under identical conditions (k_{cat} = 1.2 s^{-1} and K_m = 14 μM). The agreement in k_{cat} values indicates that the aldehyde is 'kinetically competent' to serve as an intermediate in the normal reaction pathway. It is unlikely that the aldehyde could react as fast as the natural substrate (UDPG) if it were not a true intermediate in the enzyme catalyzed reaction.

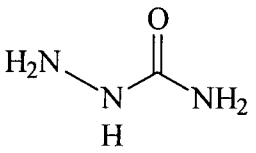


2

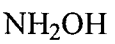
Figure 1.7 Uridine 5'-(α-D-*gluco*-hexodialdo-1,5-pyranosyl diphosphate) (**2**).

1.3.4 The Aldehyde Intermediate is Tightly Bound in the Active Site

UDPGluDH was assayed in the presence of either semicarbazide or hydroxylamine (20) (Figure 1.8). These are well-known carbonyl-trapping reagents that could form a fairly stable imine with the aldehyde intermediate. This would prevent it from undergoing the second oxidation to produce the carboxylic acid product. If the aldehyde was accessible to the bulk solvent after its formation, the use of these reagents could change the apparent stoichiometry of the enzymatic reaction; the expected ratio of 2 NADH formed from every 1 UDPG would decrease. It was found that the ratio of NADH produced to UDPG consumed remained constant at 1.9:1 over a concentration of 0 to 50 mM for each additive. This indicates that if the aldehyde is a true intermediate in the reaction pathway, it is not released into solution to any significant extent.



semicarbazide



hydroxylamine

Figure 1.8 Carbonyl-trapping reagents that could react with the aldehyde intermediate.

1.3.5 Covalent Catalysis Occurs Through Cys 260

The importance of an active site thiol in the catalytic mechanism of UDPGluDH was initially examined using uridine 5'-diphosphate chloroacetol (UDC) (Figure 1.9). This compound contains a UDP group that would allow it to bind to the enzyme in an orientation similar to the normal substrate, UDPG. It also contains an electrophilic carbon atom located three bonds away from the β-phosphate group. This carbon could be positioned in the active site of the enzyme in a location similar to the carbonyl carbon of the aldehyde intermediate. Attack by a nucleophilic residue in the active site would result in covalent bond formation between UDC and UDPGluDH.

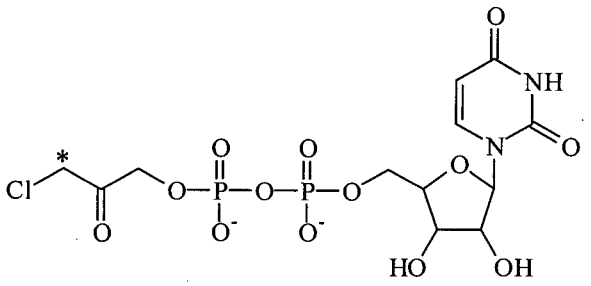


Figure 1.9 Uridine 5'-diphosphate chloroacetol (UDC). The electrophilic carbon atom that may be positioned in a location similar to the carbonyl carbon of the aldehyde intermediate is indicated with an asterisk (*).

Incubation of UDC with UDPGluDH resulted in the rapid and irreversible loss of enzymatic activity. ESI MS analysis of the inactivated enzyme showed an increase in mass correlating to the addition of one molecule of UDC minus a chlorine atom (20). The presence of competitive inhibitors of UDPGluDH slows the inactivation by UDC, indicating that the process is active site directed. This is supported by the observation that chloroacetol phosphate, a truncated form of UDC, is a much less effective inhibitor (20). When the active enzyme was denatured and treated with excess iodoacetate, two acetate units were found to be attached to the

two cysteine residues of UDPGluDH. A similar incubation using an enzyme sample that had been previously inactivated by UDC showed that only one acetate unit was added to the protein (20). This indicates that UDC inactivation was occurring through an active site cysteine thiol. UDPGluDH from *S. pyogenes* contains only one cysteine residue, Cys 260, that is strictly conserved in all known amino acid sequences. This is the best candidate for the active site residue that is modified by UDC.

The proposed role for Cys 260 was examined using the cysteine 260 to serine mutant of UDPGluDH (26). When tested under conditions similar to the wild-type enzyme for the oxidation of UDPG to UDPGA, the mutant enzyme showed less than 0.1% of the wild-type activity. Prolonged incubation of Cys260Ser UDPGluDH in the presence of UDPG produced a stable covalent adduct that was identified as UDPGA (minus H_2O) attached to the enzyme via an ester linkage. The formation of an ester intermediate at the serine residue of this mutant strongly supports the involvement of a thioester intermediate in the mechanism employed by wild-type UDPGluDH. It is very likely that both enzymes catalyze the oxidation of UDPG via a similar mechanism.

1.3.6 The Rate-Limiting Step of the Catalytic Mechanism

The accumulation of the ester intermediate in the reaction catalyzed by the Cys260Ser mutant indicates that hydrolysis of the unnatural ester linkage is extremely slow. It is also likely that in the case of wild-type UDPGluDH, hydrolysis of the thioester intermediate is rate-limiting. This agrees with earlier studies using C-6 di-deuterated UDPG as a substrate for the wild-type enzyme. If either hydride transfer step in the normal mechanism were rate-determining, the rate of oxidation of the deuterium labelled substrate should be diminished. Effectively no isotope

effect was observed (k_H/k_D = 1.1 ± 0.1) (24), suggesting that neither hydride transfer step was rate-limiting.

1.4 THE X-RAY STRUCTURE OF UDPGluDH

The biochemical experiments carried out on UDPGluDH from *S. pyogenes* have been very successful in the determination of the overall catalytic mechanism of the enzyme. However, an atomic resolution picture of the protein would be necessary to obtain a deeper understanding of this mechanism. For a protein of this size, X-ray crystallography is the only available method of obtaining such information. An X-ray structure allows the identification of interesting active site residues that may be vital to the overall catalytic mechanism. Site-directed mutagenesis may then be used to determine the exact role of such amino acids. In addition, the active site architecture and overall fold of the enzyme may reveal unsuspected evolutionary relationships to other known proteins. Such relationships may lead to further experiments that investigate possible similarities between the protein of interest and other well-studied systems.

A previous researcher in our laboratory, Dr. Robert Campbell, was successful in obtaining structural information on UDPGluDH. In particular, two X-ray structures were solved: 1) wild-type UDPGluDH with bound UDP-xylose (a competitive inhibitor of the enzyme) and NAD^+; and 2) Cys260Ser UDPGluDH with bound UDPGA and NAD(H). It was not possible to distinguish whether the oxidized (NAD^+) or reduced (NADH) form of the nicotinamide cofactor was bound to Cys260Ser; therefore, this species will be referred to as NAD(H) to reflect this uncertainty.

A more in-depth discussion of the conclusions presented here can be found in the Ph.D. thesis of Dr. Campbell (27) and the published report of these results (21).

1.4.1 Tertiary and Quaternary Structure

A ribbon representation of the structure of UDPGluDH from *S. pyogenes* is shown in Figure 1.10. The enzyme was found to be a catalytic dimer, with a total of 24 hydrogen bonds that stabilize the dimer interface. There is one residue, Arg 244, that is believed to contribute to UDPG binding in the adjacent active site, but there are no residues from the dimer partner that play a catalytic role in the enzyme.

The monomer is composed of two discrete α/β domains, each consisting of a central β-sheet surrounded by α-helices, that are connected by a long, central α-helix. The N-terminal domain consists of a typical Rossmann fold that binds NAD^+, positioning the nicotinamide ring in the active site formed at the dimer interface. The C-terminal domain is primarily responsible for the binding of the UDP group of the UDP-sugar. It positions the pyranose ring in a location that allows the transfer of a C6" hydrogen to the *si* face of the nicotinamide ring of NAD^+.

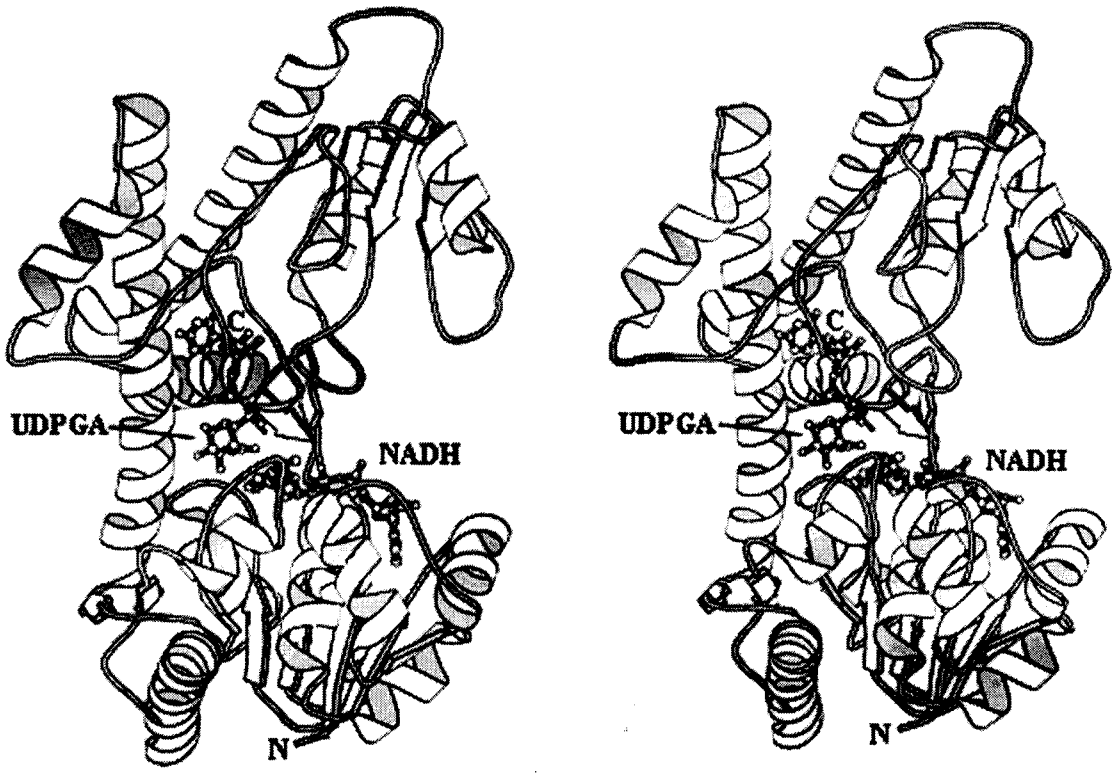


Figure 1.10 Ribbon representation of the ternary complex of the Cys260Ser UDPGluDH/UDPGA/NAD(H) monomer. UDPGA, NAD(H), and the side chain Ser260 (N-terminus of α-11) are shown in ball and stick.

1.4.2 Structural Similarity to Other Dehydrogenases

Many of the proteins that are structurally similar to UDPGluDH have dinucleotide binding sites and exhibit dehydrogenase activity. The two proteins with the greatest structural homology to UDPGluDH are short-chain-L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) (28) and 6-phosphogluconate dehydrogenase (6PGDH) (29). These enzymes share all of the major structural elements of the N-terminal domain, as well as significant portions of the central α-helix and the α-helical region of the C-terminal domain. The similarity between the enzymes ends with residue 290 of UDPGluDH. There is a remarkable conservation of identity and

conformation between two active site residues of UDPGluDH and 6PGDH: Lys 204 and Asn 208 (Lys 183 and Asn 187 in sheep liver 6PGDH). Recent studies have implicated these resides in the mechanism of 6PGDH (29, 30), and their possible roles in the catalytic mechanism of UDPGluDH will be discussed in Section 1.4.5.

1.4.3 Roles of Conserved Residues

An alignment of the primary sequences of UDPGluDH, UDPManNAcDH, and GDPManDH from a variety of organisms indicates that all secondary structural elements are conserved and there are a total of 22 strictly conserved residues. The greatest sequence diversity occurs near the C-terminus. The roles for the conserved residues can be divided into four groups:

1) Those that bind NAD^+ (6 residues).

2) Those that bind UDPG (2 residues).

3) Those that are potentially involved in catalysis or are necessary for the proper positioning of the catalytic groups (10 residues).

4) Those that are remote from the active site and substrate binding pockets; these are probably necessary for productive folding or maintaining structural integrity (4 residues).

1.4.4 Substrate Binding

The UDPG binding pocket of UDPGluDH can be divided into 2 regions: the UMP binding pocket, which is composed solely of residues from the C-terminal domain, and the glucose-1-phosphate binding pocket, consisting primarily of residues from the N-terminal

domain. NAD(H) is bound to the N-terminal domain of UDPGluDH in a typical orientation, with the nicotinamide ring in a syn conformation.

1.4.5 Sequestering of Reaction Intermediates

One of the difficulties in studying the reactions catalyzed by the UDPGluDH family of enzymes is the lack of evidence for the formation of an aldehyde intermediate. The structure of UDPGluDH indicates that the active site is deeply buried and only exposes 5 $Å^2$ of the UDP-sugar to the bulk solvent. However, the NAD^+ binding site has a solvent accessible area of 54 $Å^2$, which allows the cofactor to freely exchange during the catalytic mechanism. In order for reversible binding of the UDP-sugar to be possible, significant movement of a region of the enzyme that covers the substrate would be required.

1.4.6 Active Site Residues of UDPGluDH

Figure 1.11 illustrates the positioning of the six conserved active site residues in UDPGluDH that are contributed from both the N- and C-terminal domains and the central α-helix. The most notable of these residues is the catalytic nucleophile, Cys 260, which is located in the C-terminal domain. Asp 264 is the only other residue from this domain that is located in the active site and probably has a direct role in the catalytic mechanism.

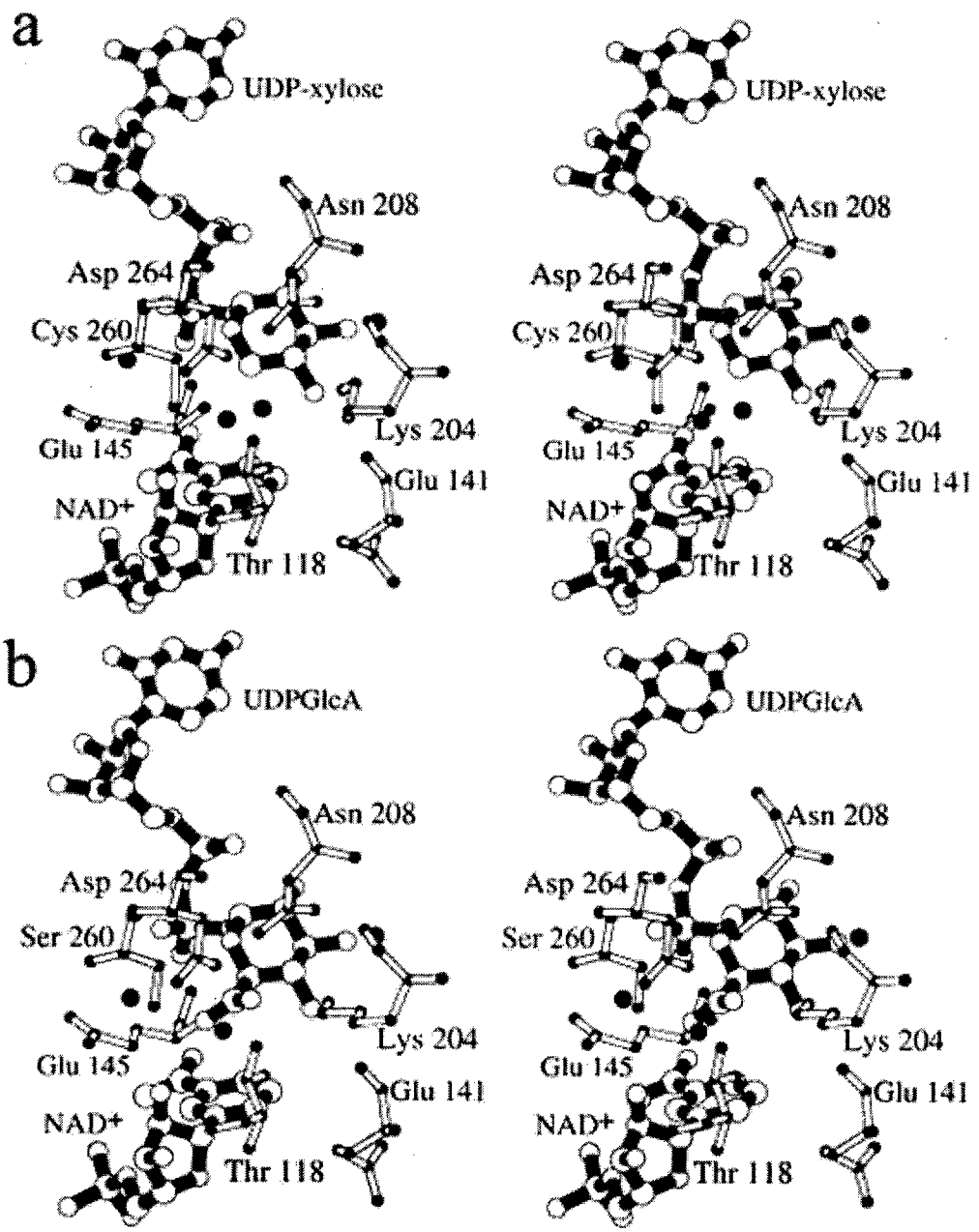


Figure 1.11 Stereo views of the active site of UDPGluDH with putative catalytic residues. **a)** The active site of wild-type UDPGluDH with bound UDP-xylose and NAD^+ truncated at the pyrophosphate bond. **b)** The active site of Cys260Ser UDPGluDH with bound UDPGA and NAD(H) (truncated at the phosphate bond). Notice that the C6" carboxylate of UDPGA occupies a position similar to a water molecule in the wild-type structure.

The N-terminal domain contributes three of these conserved active site residues. Thr 118 forms a hydrogen bond to a water molecule that may be essential for the catalytic mechanism; Glu 145 is believed to play a role in substrate specificity. Glu 141 is located beneath the nicotinamide ring of NAD(H) and forms a hydrogen bond to an active site water molecule. This activated water may be responsible for the hydrolysis of the thioester intermediate in the final step of the UDPGluDH reaction. The central α-helix contributes two strictly conserved active site residues, Lys 204 and Asn 208, which may play a role in electrostatic stabiliztion of the various intermediates formed in the enzymatic mechanism.

The first step in the reaction catalyzed by UDPGluDH involves a hydride transfer from the substrate alcohol to the NAD^+ cofactor to form an aldehyde intermediate. This type of enzymatic oxidation requires the help of a general base catalyst. The positioning of the active site residues, as revealed by the X-ray crystal structure of the enzyme, along with the known stereochemisty of the first hydride transfer, limit the number of residues that could play such a role. Two of the best candidates are Lys 204 and the water molecule that is activated through hydrogen bonding to Asp 264 (Figure 1.12 a and b).

It is generally accepted that the pK_a of free lysine in water is 10.5; if the amine is to act as a base in an enzymatic mechanism, there must be a depression in this pK_a value. Such a perturbation is usually caused by situating the lysine residue in a buried hydrophobic region of the enzyme (31) or in close proximity to other charged residues (32). Neither of these conditions applies to Lys 204 of UDPGluDH. However, since the enzyme has a relatively basic optimum pH-rate (pH 9), significant lowering of the lysine pK_a value may be unnecessary.

As mentioned in Section 1.4.2, Lys 204 is structurally analogous to Lys 183 in the enzyme 6PGDH. Recent evidence has suggested that Lys 183 is the residue responsible for

deprotonation of the substrate alcohol in the 6PGDH reaction (30). The close proximity of Lys 204 to the UDPG hydroxyl, along with the analogy to the 6PGDH mechanism, makes this residue a logical candidate for the role of general base. The alternate candidate, a water molecule activated by Asp 264, also forms hydrogen bonds to Thr 118 and a ribose hydroxyl of NAD^+. These are likely critical for the proper positioning of this potential catalytic residue.

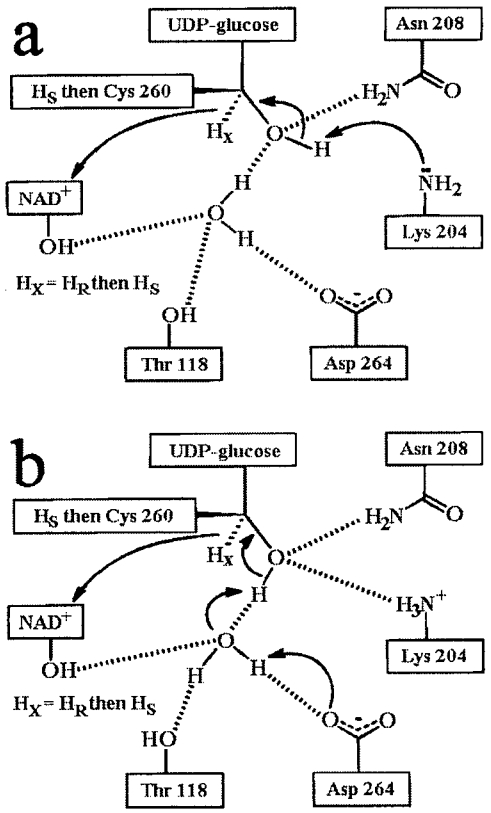


Figure 1.12 Two possible mechanisms for the 2-fold oxidation of UDPG (H_X = H_R) to the aldehyde intermediate and the thiohemiacetal intermediate (H_X = H_S) to the thioester intermediate. (a) The general acid/base catalyst is assumed to be Lys 204. (b) The general acid/base catalyst is assumed to be the water molecule that is hydrogen-bonded to Asp 264.

An electrostatically positive pocket is formed in the active site of UDPGluDH by Lys 204, Asn 208, and the water molecule coordinated to Asp 264. The catalytic group that does not act as a general base, along with Asn 208, provides electrostatic stabilization of the intermediates formed in the UDPGluDH reaction. These include the alkoxide form of the substrate alcohol,

the oxyanion of the thiohemiacetal, and all other short-lived species produced in the enzymatic mechanism. It is reasonable to propose that UDPGluDH can perform two oxidations and a hydrolysis in the same active site because the residues necessary for general acid/base catalysis and intermediate stabilization are similar in all three steps.

1.5 THE DETAILED MECHANISM OF UDPGluDH

Figure 1.13 outlines the detailed mechanism of UDPGluDH, based on all the results obtained by previous researchers in our laboratory. This mechanism is consistent with all the kinetic, mechanistic, and structural studies described in this chapter for UDPGluDH from *S. pyogenes*. It also agrees with previous studies on both the mammalian enzyme and that from other bacterial sources. In this Figure, it is assumed that Lys 204 is the catalytic base responsible for promoting the hydride transfer. The water molecule hydrogen-bonded to Asp 264 could similarly perform this role.

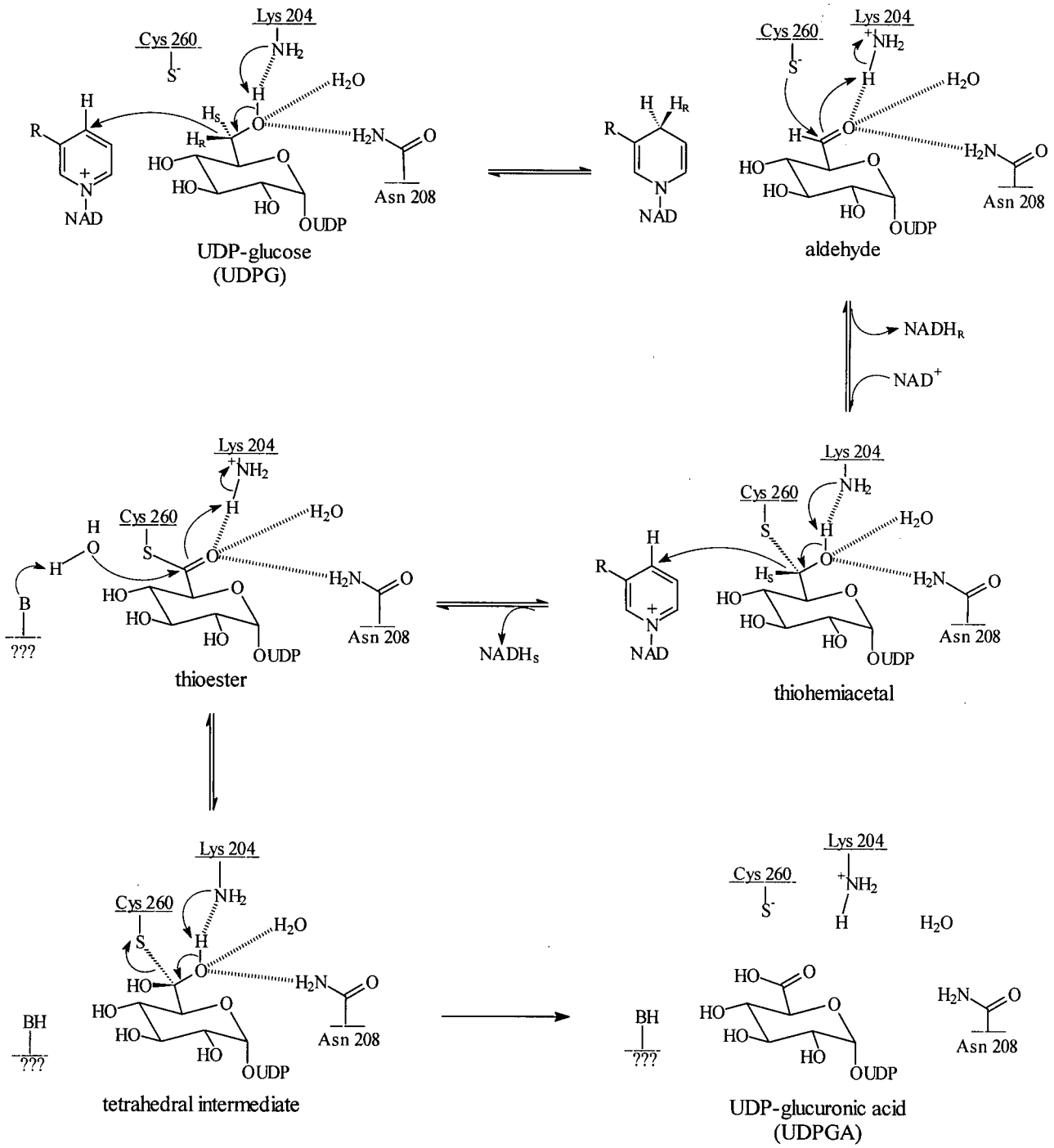


Figure 1.13 The detailed mechanism of UDPGluDH.

UDPG binds in the active site of the enzyme such that the hydroxyl group is placed in the electrostatically positive pocket formed by Lys 204, Asn 208, and a conserved water molecule. These residues assist in the removal of the hydroxyl proton through electrostatic stabilization and general base catalysis, as discussed in the previous section. Transfer of the *pro-R* hydride of the substrate to the *si* face of NAD^+ generates a tightly bound aldehyde intermediate that undergoes nucleophilic attack by the thiolate of Cys 260. This produces a thiohemiacetal intermediate that is also stabilized by the aforementioned residues. NADH then dissociates from the enzyme and another molecule of NAD^+ is bound. Oxidation of the thiohemiacetal is analogous to the first oxidation step, with a transfer of the remaining hydride to NAD^+. The second molecule of NADH dissociates, leaving the thioester intermediate that is hydrolyzed in a final rate-determining step. This occurs when an activated water molecule adds to the *re* face of the thioester to produce a tetrahedral intermediate with similar geometry to the thiohemiacetal. The collapse of this intermediate with the elimination of Cys 260 generates the product, UPDGA.

The mechanism shown in Figure 1.13 answers many specific questions regarding the UDPGluDH catalyzed two-fold oxidation of UDPG to UDPGA. However, there are many details that have yet to be revealed. Among these are the identities of the residues responsible for increasing the nucleophilicity of Cys 260, activation of the hydrolytic water molecule, and deprotonation of the substrate alcohol. Carefully designed experiments, combined with site-directed mutagenesis of the wild-type enzyme, will be the key to unravelling the remaining unanswered questions concerning UDPGluDH.

1.6 AIMS OF THIS THESIS

This thesis describes experiments designed to further investigate the mechanism utilized by UDP-glucose dehydrogenase from *Streptococcus pyogenes*. In particular, the technique of site-directed mutagenesis was used to probe the role of particular active site amino acids in the catalytic mechanism. Chapter Two describes attempts at overexpression and purification of these mutant proteins, along with the determination of kinetic constants. Chapter Three focuses on primary kinetic isotope effect experiments that further investigate the properties of these mutants.

CHAPTER 2:

THE EXPRESSION, PURIFICATION, AND KINETIC CHARACTERIZATION OF SIX MUTANTS OF UDPGluDH

2.1 INTRODUCTION

In the recent past, obtaining large quantities of homogeneous proteins for laboratory study was a difficult process. It typically involved extraction of the protein of interest from the native organism followed by a long and tedious purification procedure. The yields from this process were quite low; it was often considered a great success if only a few milligrams of pure protein were obtained. This frequently limited the proteins that were studied to those that could be found in large natural abundance.

UDP-glucose dehydrogenase is a good example of a naturally abundant enzyme because the product of its reaction, UDP-glucuronic acid, is required for a variety of critical biological processes (Section 1.1.1). The classical source of UDPGluDH, bovine liver, contains high concentrations of the enzyme because UDPGA is necessary for the efficient detoxification of waste metabolites (2). One reported procedure obtained 10 mg of UDPGluDH from 1 liter of calf liver homogenate after seven purification steps (9). The first purification of the enzyme from a bacterial source was reported in 1973; a six-step procedure yielded 14 mg of UDPGluDH from 100 g of *E. coli* cells (16). These yields are quite respectable, considering that they rely on the natural abundance of the enzyme.

An easier method of obtaining large quantities of UDPGluDH, or any other protein, would be desirable. It would also be beneficial to obtain significant quantities of proteins that are not naturally abundant. Fortunately, such procedures are now available due to advances in molecular biology, particularly in the field of recombinant DNA technology.

2.1.1 Gene Cloning and Recombinant Proteins

A great variety of enzymes are now available in quantities that were previously unimaginable. Newly developed techniques make it possible to insert a gene that encodes for a protein of interest into a host organism that is then forced to express large quantities of this protein. Subsequent purification steps are then much more efficient because the desired enzyme is present in a high proportion of the host's total cellular protein. This process begins with manipulation of the DNA containing the gene for the desired protein.

A preparation of the DNA of the organism of interest is partially digested with enzymes called restriction endonucleases that cut the DNA at specific sites to give a library of DNA fragments. Each fragment is then inserted into a piece of circular, double-stranded DNA called a plasmid, which contains the genes necessary for both replication and antibiotic resistance in a host cell. The resulting plasmid, consisting of DNA from two different sources, is known as recombinant DNA. When this is mixed with a culture of host bacterial cells, such as *E. coli*, it may be taken up and replicated in a process known as bacterial transformation. Screening for the combination of enzymatic activity and antibiotic resistance reveals bacterial colonies that contain the target gene.

Sequencing of the plasmid DNA will reveal the exact nucleotide sequence of the target gene, which can then be removed and inserted into a specially constructed plasmid called an expression vector. The expression vector contains an easily controlled promoter that only allows the desired protein to be expressed in the presence of an external stimulus. A common promoter is the T7 promoter, derived from the *lac* operon, which allows a gene to be expressed in the presence of added inducer, isopropyl-1-thio-β-D-galactopyranoside (IPTG). Bacterial cells are then transformed with the expression vector and when the inducer of the promoter is added to the

cell culture, the bacteria will begin to produce the target protein. Systems have been developed in which 40% of the cellular protein is made from the cloned gene (33).

This basic strategy was used to obtain and clone *hasB*, the *S. pyogenes* UDPGluDH gene, into the inducible p15AT7 expression vector to give the recombinant plasmid pGAC147 (4). This plasmid was provided to former members of our laboratory through a collaboration with Ivo van de Rijn of Wake Forest University. Approximately 35 mg of homogeneous UDPGluDH could be obtained from 500 mL of *E. coli* culture (20).

2.1.2 Site Directed Mutagenesis

Recombinant DNA technology now makes it feasible for researchers to create specific gene mutations *in vitro*. Mutant proteins with a single amino acid substitution can readily be produced using the technique of site directed mutagenesis. Such mutations are possible provided that a plasmid containing the gene for the protein can be prepared and the base sequence around the site to be altered is known (34). A single, specific change in the nucleotide sequence of the gene can alter the amino acid sequence of the corresponding protein in a predictable manner. Insertion of the plasmid for the mutant protein into a host organism can allow large quantities of the enzyme to be obtained and purified.

This technique is often used to probe the role of potentially important amino acid residues in the catalytic mechanism of an enzyme. Chemical modification of the enzyme can also be used to study such amino acids. For example, reagents such as iodoacetate can be used to label the thiol of a cysteine residue and make it unable to participate in the enzymatic reaction (37). However, information obtained from modification studies can be misleading. Often, modifying reagents are not specific for the residue of interest and they can cause significant change in

protein structure. For instance, the reagent may be bulky or contain multiple charges, which reduces the accessibility of the active site and interferes with substrate binding. This may lead to the false assumption that a certain amino acid is crucial to the catalytic mechanism. This was the case with histidinol dehydrogenase, an enzyme that was believed to employ a mechanism similar to that of UDPGluDH. Chemical modification studies indicated that an active site cysteine residue was essential for catalysis (36); however, subsequent mutagenesis experiments indicated that this was not the case (37, 38).

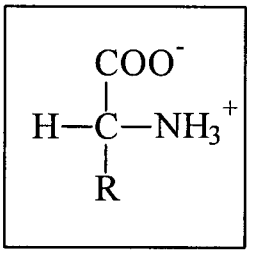
Changes in enzyme structure can be kept to a minimum when site-directed mutagenesis is used to alter a single amino acid in a protein, provided that the replacement is sterically and electronically similar to the original residue. The active site structure and overall protein fold are usually unaffected by such a mutation. Thus, any reduction in the catalytic activity of the enzyme can be reasonably assigned to the loss of the residue that was mutated. Two mutant forms of UDPGluDH from *S. pyogenes*, Cys260Ser and Cys260Ala, have been previously expressed and purified in our laboratory (24, 26). These proteins show less than 0.1 % of the wild-type activity when assayed under conditions similar to the wild-type enzyme for the oxidation of UDPG to UDPGA, indicating that Cys 260 is essential to the catalytic mechanism. In addition, the formation of a stable covalent adduct between the substrate and the mutant enzyme provided strong support for covalent catalysis in the UDPGluDH mechanism (Section 1.3.5). Thus, experiments using mutated forms of an enzyme can provide significant insight into its catalytic mechanism.

2.1.3 New Mutants of UDPGluDH

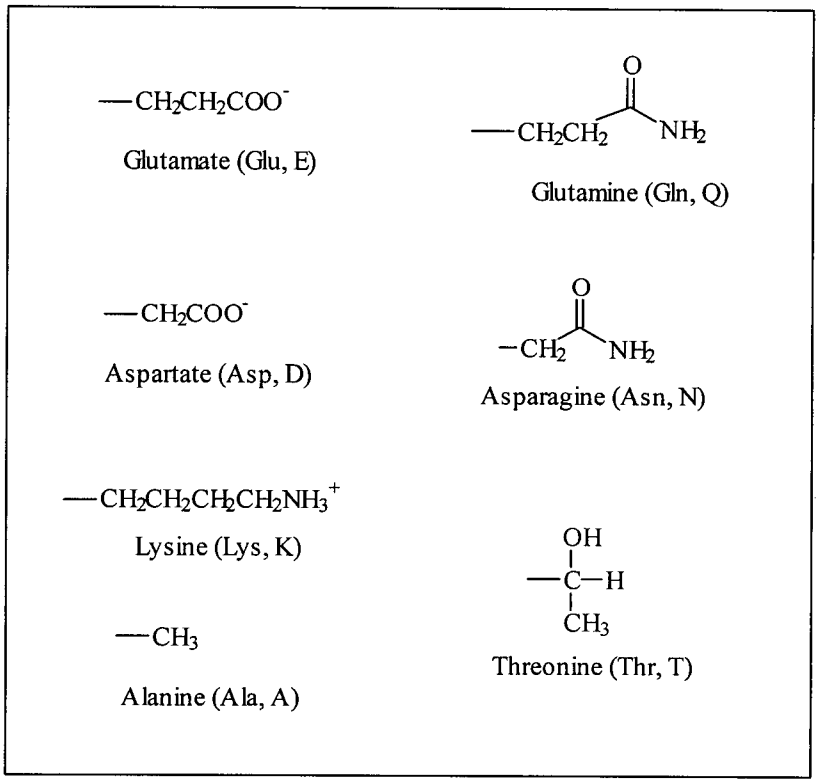
The elucidation of the crystal structure of UDPGluDH from *S. pyogenes* allowed the identification of several conserved active site residues that may play an important role in the enzyme's catalytic mechanism (Section 1.4.6). Based on this information, 6 new mutants of UDPGluDH were designed (Figure 2.1). Three of the mutants have the normal residue truncated to an alanine; this new residue is smaller than the original and should cause only minimal structural changes in the enzyme. However, the side chain of alanine is a methyl group that is unable to participate in normal enzymatic reactions within the active site. The other three mutations involve the conversion of a carboxylic acid to the corresponding amide group. In this case, the new amino acid can no longer act as an acid/base catalyst but is still capable of hydrogen bonding within the active site.

Thr118Ala	Glu141Gln
Lys204Ala	Glu145Gln
Asn208Ala	Asp264Asn

(A)



(B)



(C)

Figure 2.1 (**A**) A list of the mutants of UDPGluDH to be studied in this thesis. (**B**) The structural formula of a generic amino acid. (**C**) The R groups of the amino acids involved in the mutant enzymes.

2.2 STUDIES INVOLVING K204A, N208A, D264N

Unless otherwise stated, the procedures outlined in this section were similar for all three mutants.

2.2.1 Overexpression and Partial Purification of K204A, N208A, D264N

The plasmids for the mutant proteins were provided by Ivo van de Rijn of Wake Forest University. These were transformed into *E. coli* JM109 (DE3), grown in the presence of

chloramphenicol, and induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG). This resulted in a high level of expression of a protein with a molecular weight of approximately 45 kDa, as indicated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). In all subsequent purification steps, several precautions were taken in an attempt to maintain the stability of the enzyme. These included performing manipulations at 4 °C and adding the reducing agent dithiothreitol (DTT) to all purification buffers. In addition, 10 % glycerol was included in the purification buffers to protect the enzyme during repeated freeze/thaw cycles.

The crude cell lysate was prepared by subjecting the resuspended cell cultures to two passes through a French pressure cell, followed by the removal of insoluble cell debris by centrifugation. At this stage, analysis of both the lysate and cell debris pellet by SDS PAGE indicated that the overexpressed protein was not present in solution; rather, it had precipitated out with the cell debris (Figure 2.2). The regular purification procedure was no longer applicable. It is likely that the protein that was overexpressed had been formed as insoluble inclusion bodies (see next Section).

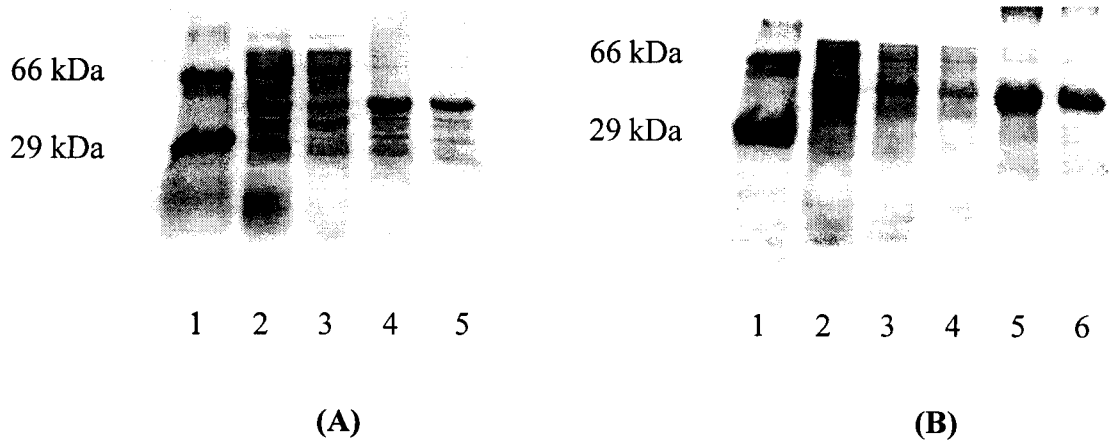


Figure 2.2 (A) SDS PAGE of D264N. *Lane 1*, molecular weight markers, *lanes 2 & 3*, lysate after cell lysis; *lanes 4 & 5*, solid pellet after cell lysis. **(B)** SDS PAGE of N208A. *Lane 1*, molecular weight markers; *lanes 2, 3, 4*, lysate after cell lysis; *lanes 5 & 6*, solid pellet after cell lysis. Similar results were obtained with K204A.

2.2.2 Inclusion Body Formation

High level expression of recombinant proteins in *E. coli* often results in the formation of large intracellular aggregates known as inclusion bodies. These are insoluble in the normal purification buffer and contain inactive protein (39). There are several methods of dealing with inclusion body formation during protein overexpression. Often the problem can be avoided altogether by switching to expression systems that use yeast or mouse cells (33). If *E. coli* must be used, slight alterations in the growth conditions can make a big difference to the overall degree of soluble protein obtained. There have been increasingly successful examples of expression of soluble recombinant proteins at low growth temperatures (40). This may be due to a decreased rate of protein synthesis, which could favor protein folding and minimize aggregation. Another factor that appears to be important is the amount of IPTG used. Low IPTG concentration causes incomplete or partial induction, which also leads to a lower rate of protein synthesis and a greater degree of protein folding (40).

While initially considered to be problematic, the formation of inclusion bodies can be advantageous for the production of recombinant proteins. Under appropriate conditions, the recombinant protein deposited in inclusion bodies can amount to over 50 % of the total cellular protein. Because the inclusion bodies have a relatively high density, they can be isolated from many cellular proteins by centrifugation. The purity of the resulting solid pellet can be as high as 90 % for the target protein (in a denatured form) (41).

To obtain the desired protein in an active form, the purified inclusion bodies must be solubilized by strong denaturants such as 6 M guanidinium-HCl or 8 M urea. Next, excess denaturants must be removed by either dialysis or dilution. This is usually performed at low protein concentrations in order to minimize aggregation. The degree of refolding is strongly

dependent on both pH and temperature, and the time required for complete renaturation may extend over a range of seconds to days. The presence of low molecular weight additives, such as L-arginine, may also have a marked effect on the yield of renaturation. The optimal refolding conditions vary greatly from one protein to another (41).

While the solubilization of inclusion bodies can provide a relatively pure protein sample, the process of optimizing the renaturation conditions for a given protein can be quite time-consuming. Some proteins will resist refolding regardless of what conditions are attempted. Another problem arises when one is dealing with mutant forms of an enzyme. The degree of protein renaturation is usually monitored using an assay for enzymatic activity. A mutant enzyme may have an extremely low degree of activity in this assay, even when it is properly folded. In this case, it would be ideal to determine the conditions under which denatured wild-type enzyme can be refolded, and then apply these to the denatured mutant. Comparison of the circular diochroism (CD) spectra of native and mutant proteins may give an indication of whether refolding has occurred (42).

2.2.3 Attempts to Overcome Inclusion Body Formation

Initially, attempts to express the mutant proteins involved growing *E. coli* cultures at 37 °C both before and after induction with IPTG. In an attempt to reduce the production of inclusion bodies with the D264N and N208A mutants, the growing temperature was lowered to 30 °C after IPTG addition. The cultures were then allowed to grow for a further 5 – 6 hours, as opposed to 3 – 4 hours at 37 °C. However, there was no significant improvement in the amount of soluble protein obtained.

Next, it was decided to lower the IPTG concentration from 400 μM to 100μM. This led to slight improvements in the amount of soluble protein for all three mutants. Further reduction in the concentration, sometimes as low at 10 μM, didn't reduce the amount of protein in inclusion bodies. With D264N and N208A, it was found that a combination of lower temperature and lower IPTG concentration led to slight improvements in the amount of soluble protein. But in all cases, centrifugation produced a solid pellet that contained a large amount of protein with a molecular weight of approximately 45 kDa, as indicated by SDS PAGE analysis. This protein accounted for a high percentage of the total protein present in the solid. An attempt was then made to obtain the mutant enzymes from the inclusion bodies.

The pellet containing D264N UDPGluDH inclusion bodies was washed with a buffer containing 2 M urea and then resuspended in a buffer with 8 M urea. The solution was incubated for several hours at 37 °C, with occasional vortexing, and solids were removed by centrifugation. The lysate was then subjected to several rounds of dialysis/centrifugation in an attempt to refold the denatured protein. Analysis by SDS PAGE indicated that the overexpressed protein remained in solution, but no enzymatic activity could be detected even at very high enzyme and substrate concentrations. A similar procedure was followed for the K204A mutant and gave similar results.

2.2.4 Future Studies

The three mutated residues discussed in this section are involved in stabilizing the intermediates in the UDPGluDH mechanism through the formation of an electrostatically positive pocket in the active site. As well, either Lys 204 or a water molecule hydrogen bonded to Asp 264 acts as a general base to deprotonate the substrate alcohol (Section 1.4.6). The

K204A and D264N mutant enzymes will probably provide much insight into the catalytic mechanism utilized by UDPGluDH.

It is likely that all three of the mutants discussed will have a much lower activity than the wild-type enzyme. This will make it necessary to obtain large quantities of the proteins for determination of the kinetic constants. The most likely method of achieving this will be solubilization of the inclusion bodies followed by renaturation of the mutant protein. A starting point for this process would be to denature the wild-type enzyme and determine the conditions under which it is successfully refolded. Application of these conditions to the mutant enzymes may be successful in renaturing the proteins, even if this cannot be monitored through the usual UDPGluDH activity assay. If it were possible to renature the mutant enzymes from inclusion bodies, the protein would likely be quite pure, as most other cellular proteins would be soluble in the cell lysate. It may be possible to completely purify the enzymes using only one anion-exchange column, as opposed to two such columns required for wild-type UDPGluDH.

Further alterations in the growing conditions may result in less inclusion body formation; attempting to grow the proteins in another host organism, such as yeast, may also reduce the problem of protein aggregation.

One interesting experiment to be attempted once the proteins have been obtained in significant quantities involves the Lys204Ala mutant. This mutation has left a gap in the active site, since the alanine residue is missing three $-CH_2$ groups and the terminal $-NH_2$. It is postulated that including a small amine, such as ethylamine, in the assay buffer may help to "rescue" any lost activity. The small molecule may be able to diffuse into the active site and perform the same role as the original lysine residue. If this recovery of activity were possible

when using a methylated quaternary salt of ethylamine, it would be evidence that the primary role of Lys 204 was electrostatic and not to act as a general base.

2.3 STUDIES INVOLVING T118A, E141Q, E145Q

Unless otherwise stated, the procedures outlined in this section were similar for all three mutants.

2.3.1 Overexpression and Purification of T118A, E141Q, E145Q

The plasmids for these mutant proteins were also provided by Ivo van de Rijn of Wake Forest University. The overexpression conditions and buffer additives were the same as those outlined in Section 2.2.1. However, after two passes of the resuspended cell culture through a French pressure cell and removal of the solids by centrifugation, it was determined that significant quantities of these mutant enzymes remained in the cell lysate. This solution was then partially purified by loading it onto a diethylaminoethylcellulose (DE52) weak anion-exchange column and eluting it with a linear gradient of NaCl. This step removed the strong-binding negatively charged impurities from the crude lysate. SDS PAGE analysis was used to determine which fractions contained the overexpressed protein and these were then pooled, concentrated, and desalted. The resulting protein was further purified by loading it onto a HiTrapQ Sepharose High Performance strong anion-exchange column and eluting with a linear gradient of NaCl. Again, fractions containing the overexpressed protein were determined by SDS PAGE analysis and these were pooled, frozen in liquid nitrogen, and stored at –78 °C.

The protein obtained using this method appeared to be homogeneous as judged by SDS PAGE analysis; a single protein band with a molecular weight of approximately 45 kDa was

observed for each mutant (see Figure 2.3). The purification procedure gave approximately 20 mg of protein per litre of culture, although this amount was lower for T118A.

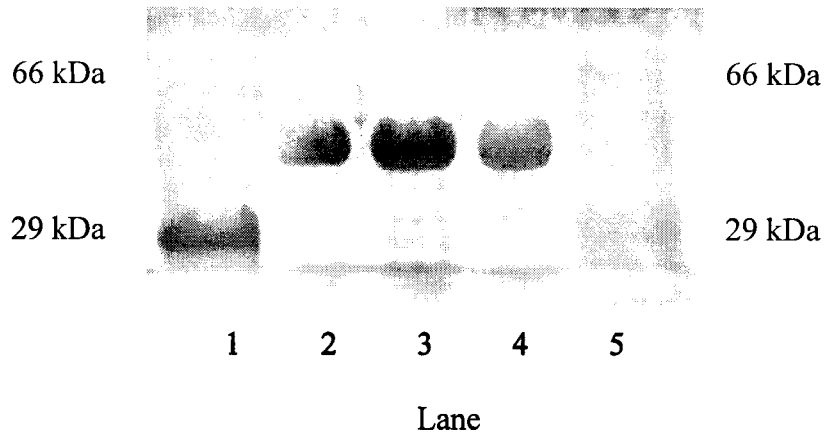


Figure 2.3 SDS PAGE of each mutant protein that was purified to homogeneity. A similar amount of protein was loaded in each lane. *Lane 1*, molecular weight markers; *lane 2*, T118A; *lane 3*, E141Q; *lane 4*, E145Q; *lane 5*, molecular weight markers.

The molecular weights of the resulting proteins were determined using electrospray ionization mass spectrometry (ESI MS). The molecular weights of both glutamate mutants were expected to be indistinguishable from that of wild-type UDPGluDH; this was confirmed by ESI MS (wild-type: 45495 Da; E145Q: 45495 Da; E141Q: 45488 Da; expected mass of 45487 Da for wild-type and 45486 Da for E141Q and E145Q). The molecular weight of T118A was found to be 45466 Da, compared to an expected mass of 45457 Da.

2.3.2 Kinetic Constants for a 2-Substrate System

To understand the terms and conventions used in describing the kinetic parameters of a 2-substrate system, a brief outline of single substrate enzyme kinetics is required. A more detailed discussion can be found in reference 34.

The critical feature of the Michaelis-Menten model is that a specific ES complex is a necessary intermediate in catalysis. The model proposed is as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

An enzyme (E) and its substrate (S) are in rapid equilibrium between the free species (E + S) and the bound complex (ES). Catalytic turnover of the ES complex then occurs as a first order process. Under this model, there are several conditions that must be met:

1) Reaction is under initial velocity conditions, so $[S] = [S]_0$, where $[S]_0$ is the initial concentration of substrate.

2) There is no product present, so k_2 is essentially irreversible.

3) $[S] >> [E]_T$, where $[E]_T$ is the total concentration of bound and free enzyme.

4) There are no allosteric or cooperative effects.

If steady-state conditions are assumed in this model (meaning that [ES] is constant), then the following equation can be derived to relate the rate constants of the individual steps to the overall substrate and enzyme concentrations:

$$v_0 = \frac{V_{max}[S]}{K_m + [S]} \quad \text{where } K_m = \frac{k_{-1} + k_2}{k_1} \quad \text{and } V_{max} = k_2[E]_T \qquad \textbf{Eq. 1}$$

The constant K_m is called the Michaelis constant and is equal to the substrate concentration at which the reaction rate is half its maximum value. The value of K_m can be a

very complicated function of rate constants in more complex systems, but it is most easily understood as the apparent dissociation constant for all ES complexes in the steady state.

$$K_m = \frac{[E][S]}{\sum[ES]} \qquad \textbf{Eq. 2}$$

V_{max} is the maximal velocity for the enzymatic reaction. Dividing V_{max} by the total enzyme concentration gives the first order rate constant for the overall reaction, k_{cat}. This is equal to the number of moles of substrate converted into product per unit mole of enzyme, commonly referred to as the turnover number.

$$k_{cat} = \frac{V_{max}}{[E]_T} \qquad \textbf{Eq. 3}$$

It is convenient to transform the Michaelis-Menten equation into one that gives a straight-line plot. This can be done by inverting both sides of Eq. 1 to give an equation of the form y = mx + b, where m is the slope and b is the y-intercept.

$$\frac{1}{v_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \qquad \textbf{Eq. 4}$$

A plot of $1/v_0$ vs $1/[S]$ is called a Lineweaver-Burk plot. It yields a straight line with a slope of K_m/V_{max}, y-intercept of $1/V_{max}$, and x-intercept of $-1/K_m$.

This treatment of enzyme kinetics deals with the simplest model system in order to introduce the basic concepts involved. In more complicated multisubstrate systems, the interpretation of the rate equations becomes much more difficult. A general and non-mathematical method for the interpretation of enzyme kinetics has been devised by Cleland (43, 44, 45); this was introduced in Section 1.3.1 using the UDPGluDH reaction as an example of a

Bi Uni Uni Bi ping pong mechanism. A description of this method is beyond the scope of this work. However, it will suffice to state that every kinetic mechanism with multiple substrates and products will have a distinguishing set of initial velocity and product inhibition patterns. The experimental procedure to obtain these patterns involves measuring the initial velocity of the reaction while varying the concentration of one substrate in the presence of a changing fixed concentration of a second substrate or inhibitor. The data is plotted in the form of a double reciprocal plot ($1/v_0$ versus 1/[variable substrate]) that will result in a set of lines that may either not intersect (parallel), intersect on the y-axis, or intersect to the left of the y-axis. This pattern can be used to determine the order of substrate binding and product release. A replot of the intercepts versus the reciprocal of the concentration of each changing fixed substrate can be used to reveal the K_m and k_{cat} values for the system.

2.3.3 Kinetic Assay for UDPGluDH Activity

The overall reaction of UDPGluDH, as shown in Figure 2.4, is effectively irreversible and one of the products, NADH, shows a characteristic absorption at 340 nm. This makes it possible to follow the progress of the reaction by continuously monitoring the absorbance at 340 nm.

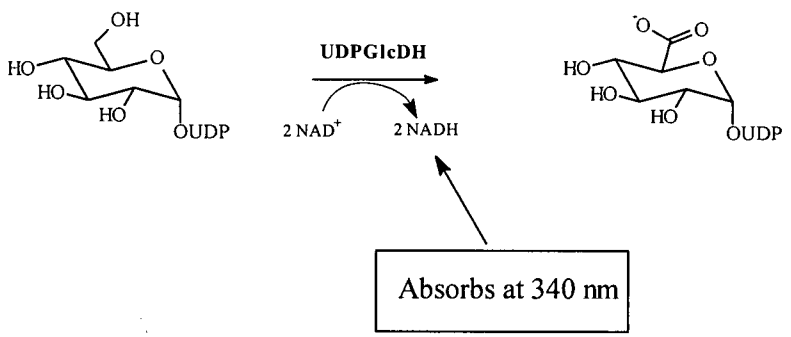


Figure 2.4 The overall mechanism catalyzed by UDPGluDH.

To determine the kinetic constants for each of the mutant proteins, it was necessary to determine the initial rates of the enzymatic reaction at varying concentrations of both UDPG and NAD^+, as outlined in the previous section. This was performed with the E141Q and E145Q mutant proteins and resulted in the plots shown in Figures 2.5 – 2.8. It should be noted that the variable substrate refers to the species for which the reciprocal of the concentration is plotted along the x-axis. The changing fixed substrate refers to the species whose concentration remains fixed on each line of the double reciprocal plot.

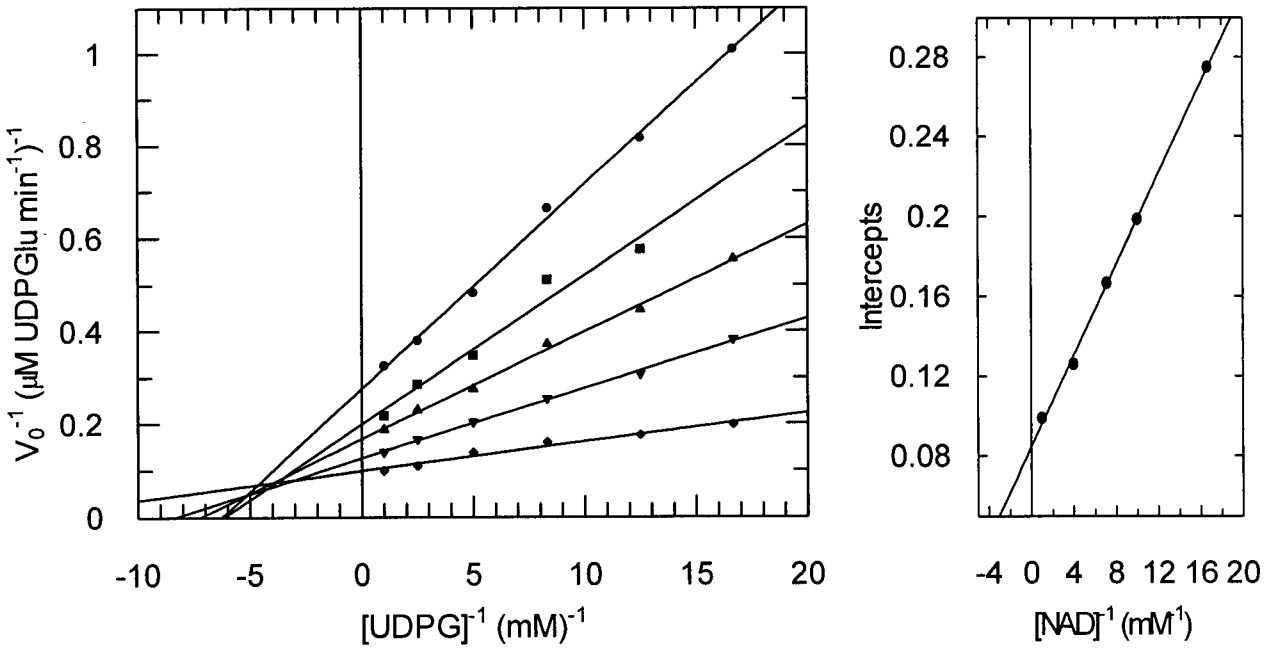


Figure 2.5 Initial velocity pattern for **E141Q** with UDP-glucose as the variable substrate. NAD^+ concentrations are 60 μM (•), 100 μM (■), 140 μM (▲), 250 μM (▼), and 1 mM (♦). The graph on the right is a replot of the y-intercepts versus $[NAD^+]^{-1}$.

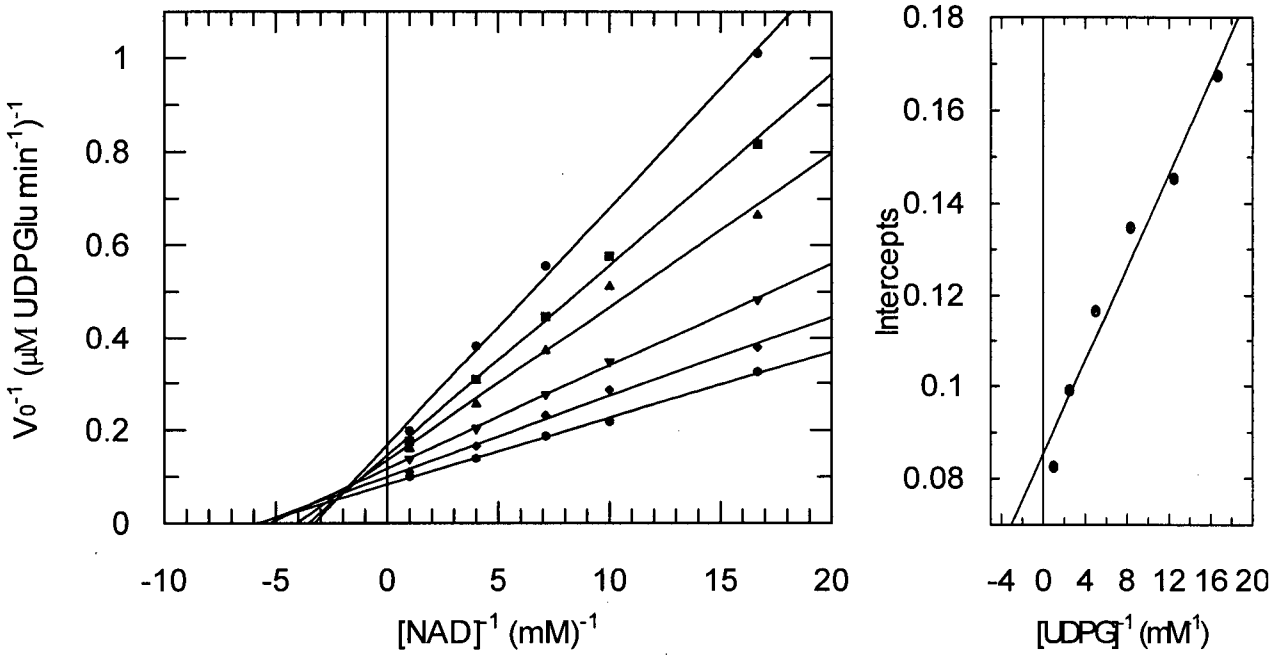


Figure 2.6 Initial velocity pattern for **E141Q** with NAD^+ as the variable substrate. UDP-glucose concentrations are 60 μM (•), 80 μM (■), 120 μM (▲), 200 μM (▼), 400 μM (♦), and 1 mM (⬢). The graph on the right is a replot of the y-intercepts versus [UDP-glucose]$^{-1}$.

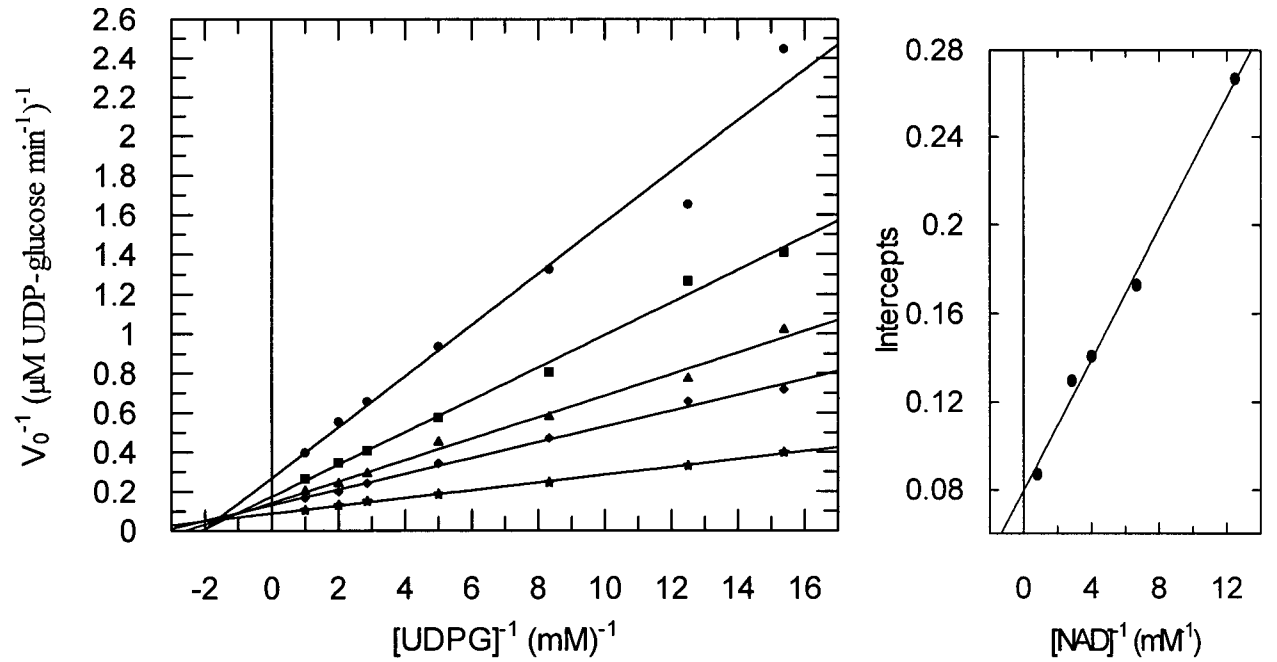


Figure 2.7 Initial velocity pattern for **E145Q** with UDP-glucose as the variable substrate. NAD^+ concentrations are 80 μM (•), 150 μM (■), 250 μM (▲), 350 μM (♦), and 1.25 mM (★). The graph on the right is a replot of the y-intercepts versus $[NAD^+]^{-1}$.

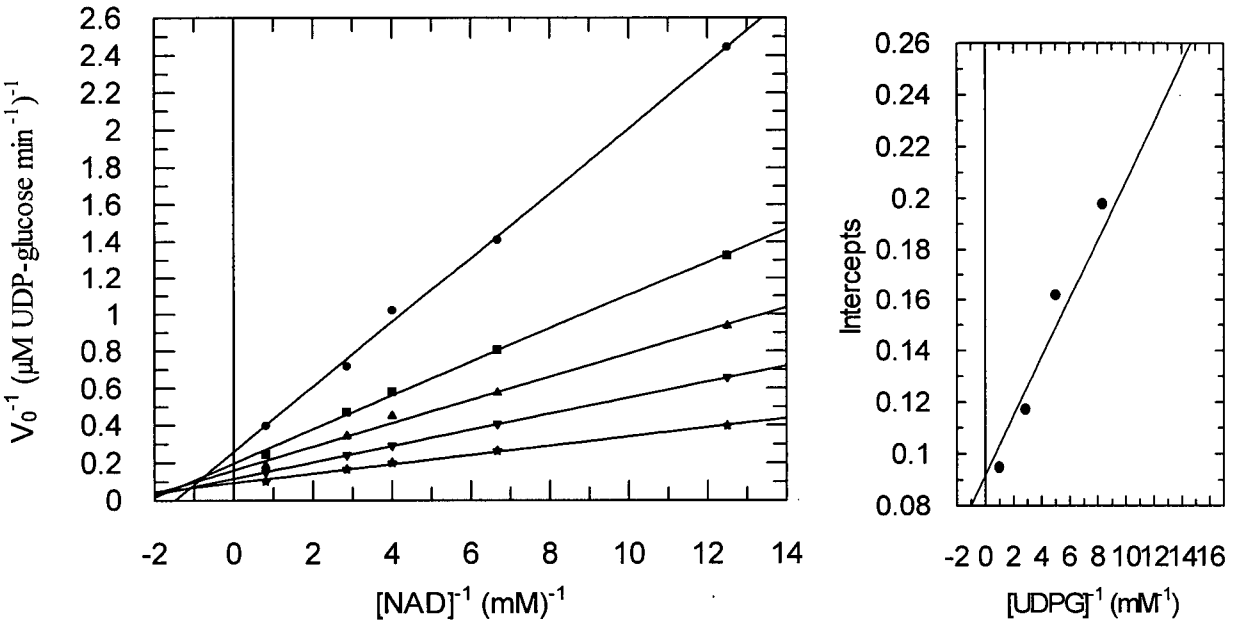


Figure 2.8 Initial velocity pattern for **E145Q** with NAD^+ as the variable substrate. UDP-glucose concentrations are 65 μM (•), 120 μM (■), 200 μM (▲), 350 μM (▼), and 1 mM (★). The graph on the right is a replot of the y-intercepts versus $[UDP\text{-}glucose]^{-1}$.

Ideally, this method would also have been used to obtain the kinetic constants for T118A. However, this mutant was less active than the glutamate mutants and required a greater amount of protein for each assay point. The total amount of protein needed was greater than could be obtained. To determine the kinetic parameters for this mutant, a high concentration of one substrate was used at changing concentrations of the other substrate. A plot of the V_0 values obtained versus concentration of the variable substrate indicated that saturation was reached. The corresponding inverse plots revealed the K_m and k_{cat} values of T118A (Figures 2.9 and 2.10)

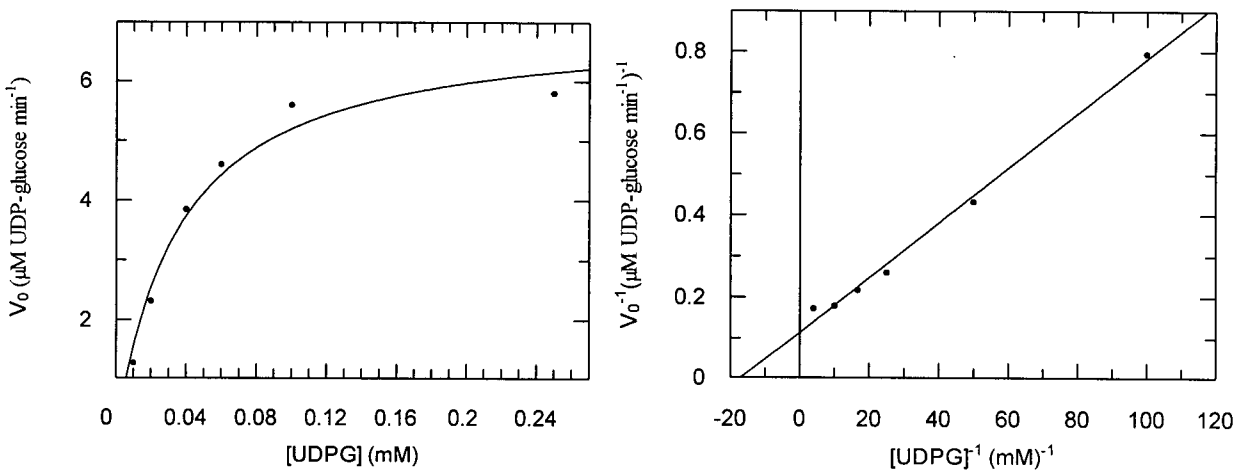


Figure 2.9 Kinetic data for **T118A** with UDPG as the variable substrate. [NAD^+] was 4 mM for each point. The inverse plot on the right was used to obtain the K_m and k_{cat} values for the mutant enzyme.

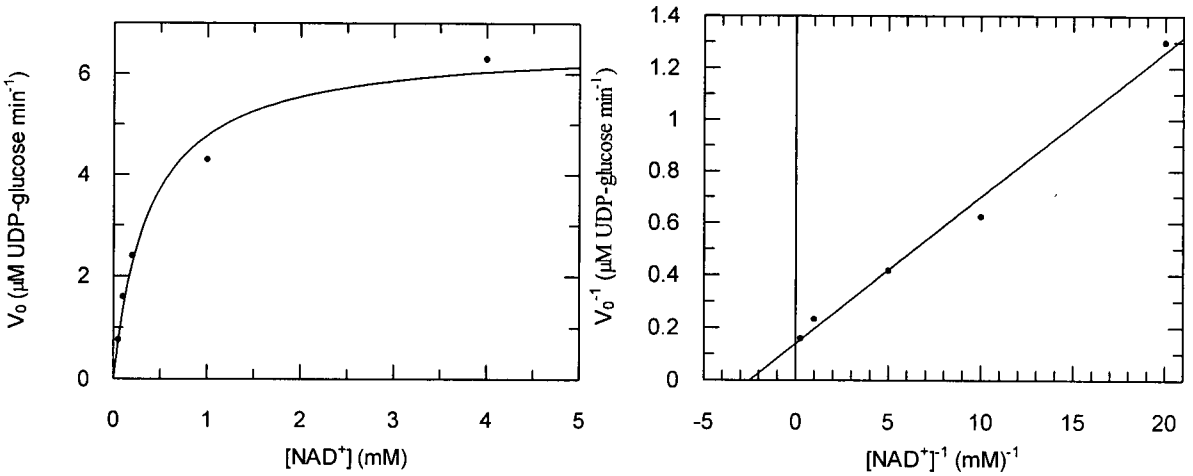


Figure 2.10 Kinetic data for **T118A** with NAD^+ as the variable substrate. [UDPG] was 0.5 mM for each point. The inverse plot on the right was used to obtain the K_m and k_{cat} values for the mutant enzyme.

A summary of the K_m and k_{cat} values for each of the purified mutants can be found in Table 2.1.

Mutant Protein	Specific Activity (units/mg)	K_m for UDPG (μM)	K_m for NAD^+ (μM)	k_{cat} (s^{-1})
T118A	0.011	59 ± 9	400 ± 100	0.011 ± 0.003
E141Q	0.16	60 ± 9	135 ± 7	0.14 ± 0.02
E145Q	0.21	125 ±24	187 ± 20	0.20 ± 0.04
Wild-type UDPGluDH	1.5 (22)	20 ± 4 (26)	65 ± 6 (26)	1.8 ± 0.1 (26)

Table 2.1 A summary of the specific activities and kinetic constants of T118A, E141Q, E145Q.

2.3.4 Discussion

This section described the successful expression and purification of three of the mutants of UDPGluDH – T118A, E141Q, and E145Q. Inclusion body formation, while still occurring to a certain extent, was less of a problem with these proteins than the mutants described in the previous section. Even though inclusion body formation was still occurring, significant quantities of soluble protein were produced in the *E. coli* cultures, allowing purification to proceed from the cell lysate. A two-step procedure was used, involving both a weak and a strong anion exchange column. Each resulting protein appeared to be homogeneous, as indicated by SDS PAGE, and their mass spectra were consistent with the expected values. The yield of protein in each case was lower than that reported for the wild-type enzyme, which may be the result of inclusion body formation

Kinetic parameters were determined for each mutant. The values obtained for E141Q and E145Q were within an order of magnitude of the wild-type values. This indicates that these

glutamate residues are not of vital importance to the catalytic mechanism of UDPGluDH. If they were essential for the binding of substrate, one would expect a K_m value that was significantly higher than that of the wild-type enzyme. If they were needed for catalysis to occur, one would expect a greatly decreased k_{cat} value. Since both parameters were only slightly altered, one can conclude that mutating these residues caused only minor perturbations within the active site of the enzyme.

A more interesting result was obtained with the T118A mutant. While the K_m values remained essentially unchanged from that of the wild-type enzyme, the k_{cat} value was decreased by 100-fold. This is a more substantial change than was observed with the glutamate mutants, indicating that Thr 118 is more important to the catalytic mechanism. Since the k_{cat} value was more affected by the mutation than the K_m values, it appears that Thr 118 plays a more important role in catalysis than in substrate binding.

2.4 MATERIALS AND METHODS

2.4.1 General Procedures

The plasmids for all six mutant proteins were provided by Ivo van de Rijn at Wake Forest University, Winston-Salem, North Carolina. These plasmids all contain the gene for chloramphenicol resistance and can be transformed into competent *E. coli* JM109 (DE3) and induced with IPTG. Plasmid DNA was prepared using the Promega WIZARD Mini Plasmid Preps kit. UDP-glucose and NAD^+ were purchased from Sigma; tryptone, yeast extract, and agar were purchased from Difco. Protein concentrations were determined by the method of Bradford (46), using bovine serum albumin (BSA) as the standard. Both the protein assay solution and the standard protein were purchased from Bio-Rad. All other buffers and chemicals were obtained

from Sigma, Aldrich, or Fisher Scientific. Unless otherwise noted, all protein manipulations were performed at 4 °C.

Protein purity was determined using 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) stained with Coomassie blue as described by Laemmli (47). Carbonic anhydrase (29 kDa) and BSA (66 kDa) were used as molecular weight standards.

All enzyme kinetic assays were recorded on a Varian Cary 3E UV-Visible spectrophotometer equipped with a circulating water bath. A unit of enzyme activity is defined as the amount of enzyme necessary to produce 2 μM NADH/min at 30 °C. The extinction coefficient of NADH was taken as 6220 M^{-1} at 340 nm. The program Grafit (Erithicus Software Ltd.) was used for the construction of all plots and the determination of the least squares linear fit to the kinetic data. ESI MS was performed by Mr. Shouming He on a Perkin-Elmer Sciex API300 electrospray mass spectrometer.

2.4.2 Specific Procedures

Expression of K204A, N208A, D264N

The plasmids for the mutant proteins were transformed into calcium chloride competent *E. coli* JM109 (DE3) by heat shocking for 2 minutes at 42 °C and then inoculated onto LB-agar plates containing 50 μg/mL chloramphenicol. Plates were incubated overnight at 37 °C and then stored at 4 °C for a maximum of 3 days. Single colonies of transformed *E. coli* were used to inoculate 500 mL of autoclaved TYPG media (8 g of tryptone, 8 g of yeast extract, 2.5 g of NaCl, 1.25 g of K_2HPO_4, and 2.5 g of D-glucose) containing 50 μg/mL chloramphenicol. The cell culture was allowed to shake at 37 °C and 230 rpm until the absorbance at 600 nm was

between 0.5 – 0.7. IPTG was added at a final concentration of 0.1 mM and the bacteria were allowed to grow for a further 3-4 hours at 37 °C and 230 rpm. The cells were then harvested by centrifugation for 20 minutes at 5000 rpm (Sorvall GSA rotor) and the cell pellet was stored at –78 °C.

The cell pellet was thawed rapidly with warm water and resuspended in 10 mL of cold buffer A (50 mM triethanolamine-HCl (Trien-HCl) pH 8.7, containing glycerol (10 % by volume), DTT (2 mM), pepstatin (1 mg/L), aprotinin (1 mg/L), and phenylmethylsulfonyl fluoride (PMSF, 1.5 mM)). The resuspended cells were lysed by two passes through a chilled French pressure cell at 20 000 psi and solids were separated by centrifugation at 6000 rpm for 40 minutes. SDS PAGE analysis of the resulting lysate and solid indicated that most of the overexpressed protein was present in the solid pellet.

Several changes in the growth conditions were attempted, as described in Section 2.2.3, to reduce the amount of protein in inclusion bodies. None of these were greatly successful and will not be described in greater detail.

Solubilization and Renaturation of D264N

The solid pellet obtained after cell lysis and centrifugation was resuspended in 50 mM Tris-HCl pH 8.2 containing 2 M urea and 2 mM DTT. This mixture was centrifuged at 5000 rpm for 20 minutes and the procedure was repeated. The resulting solid was resuspended in 50 mM Tris-HCl pH 8.2 containing 8 M urea and 2 mM DTT, and this mixture was incubated for 2 hours at 37 °C with occasional vortexing. The solution was centrifuged and insoluble debris was discarded. The lysate (30 mL) was then dialyzed against 500 mL of 50 mM Trien-HCl pH 8.7 containing 2 mM DTT at 4 °C overnight; solution was then centrifuged to remove insoluble

debris and the dialysis was continued with a fresh buffer solution. This procedure was repeated several times. SDS PAGE indicated that an overexpressed protein with a molecular weight of 45 kDa was present in solution. However, no activity was detected in the usual UDPGluDH activity assay, even at very high substrate and enzyme concentrations. This indicated that the protein was not refolded properly. Attempts to further purify this protein were unsuccessful due to the fact that the protein stuck to a column of anion exchange resin and didn't elute until a high salt concentration was used.

Solubilization and Renaturation of K204A

The procedure described was performed using two solutions of different pH. All steps outlined were identical for each solution except for the pH of the buffer involved.

The cell pellet after lysis and centrifugation was resuspended in 50 mM Trien-HCl, pH either 7.3 or 8.3, containing 2 mM DTT. This suspension was centrifuged at 6000 rpm for 20 minutes and procedure was repeated. The resulting solid pellet was then resuspended in 50 mM Trien-HCl containing 2 mM DTT and 8 M urea. These solutions were incubated at 37 °C for a period of 1.5 hours with occasional vortexing. Centrifugation at 6000 rpm for 20 minutes removed any insoluble debris; the lysate was then dialyzed overnight at 4 °C against 500 mL of 50 mM Trien-HCl, at the same pH as in the solubilization step and containing 2 mM DTT and 2 M urea. The resulting solution was dialyzed against buffer containing no urea for several hours at 4 °C. The solution was then centrifuged to remove solid debris and redialyzed. As before, SDS PAGE indicated that a protein of molecular weight 45 kDa was predominant in solution, but no activity could be detected. This was true regardless of the pH. It should be noted that during

the dialysis, there was more solid formation in the pH 7.3 buffer. This may be an indication that the protein is refolding but forming new aggregates.

Expression and Purification of T118A, E141Q, E145Q

The procedure for the transformation and expression of these mutants was the same as that described for K204A, N208A, and D264N, up to the point of cell lysis and centrifugation. SDS PAGE analysis indicated that significant quantites of T118A, E141Q, and E145Q remained in the cell lysate. This was then diluted with buffer A to three times the original volume and loaded onto a 50 mL column of diethylaminoethylcellulose (DE52) that had been preequilibrated with buffer A. The column was washed with buffer A (50 mL) and then eluted with a linear gradient of NaCl in buffer A (0 to 300 mM over 200 mL). SDS PAGE was used to determine the fractions containing the mutant enzyme. These were then pooled and concentrated to approximately 10 mL using Millipore Ultrafree centrifugal concentrators (10 kDa molecular mass cut-off) and dialyzed overnight against 1 L of buffer B (50 mM Trien-HCl pH 8.7, containing 2 mM DTT and glycerol (10 % by volume)). The resulting solution was passed through a 0.45 μm filter (Sterile Acrodisc Syringe Filters), frozen in liquid nitrogen, and stored at –78 °C.

The DE52 purified protein solution was quickly thawed in warm water and applied (approximately 20 to 25 mg of total protein in each injection) to a HiTrapQ Sepharose High Performance ion exchange column (5 mL) that had been preequilibrated with filtered and degassed buffer B. The column was washed with buffer B (50 mL) and then eluted with a linear gradient of NaCl in buffer B (100 – 200 mM over 100 mL for E141Q and E145Q and 0 – 200 mM over 100 mL for T118A). SDS PAGE was used to determine the fractions containing the

mutant protein. These were pooled, frozen in liquid nitrogen, and stored at –78 °C. The purity of the resulting enzyme was assessed using SDS PAGE. For each mutant, a single protein of approximately 45 kDa accounted for most of the protein in the final solution.

Kinetic Assay of UDPGluDH

All assays were performed in 50 mM Trien-HCl pH 8.7 containing 2 mM DTT (1 mL total volume) and were preincubated at 30 °C for 5 minutes. Initial velocity studies to obtain the kinetic constants were performed by including varying concentrations of both UDPG and NAD^+ in the assay buffer. The reaction was initiated by the addition of the mutant protein (approximately 50 μg for E141Q and E145Q and 500 μg for T118A, typically in a volume not exceeding 50 μL) and the solution was thoroughly mixed. Initial velocities were determined by following the increase in absorbance at 340 nm during the first 60 seconds after initiation with the enzyme. The slopes were calculated using a least squares analysis with Cary 3 software version 3.0. The substrate concentration ranges varied with the different mutants; they were as follows:

E141Q: [UDPG] and [NAD^+]: 0.06 – 1 mM

E145Q: [UDPG]: 0.065 – 1 mM; [NAD^+]: 0.08 – 1.25 mM

T118A: [UDPG]: 0.01 – 4 mM; [NAD^+]: 0.05 – 4 mM

The calculated initial velocities were plotted as a function of substrate concentration and the kinetic parameters were determined using double reciprocal plots, as described in Section 2.3.2.

CHAPTER 3:

PRIMARY KINETIC ISOTOPE STUDIES ON THE T118A, E141Q, AND E145Q MUTANTS OF UDPGluDH

3.1 INTRODUCTION

It is frequently observed that the rate of a reaction in which a carbon-deuterium bond is cleaved is several times slower than that of the corresponding reaction in which a carbon-hydrogen bond is cleaved (48). This is known as a primary kinetic isotope effect, and one can be confident of this designation if the overall rate is slowed by a factor of two or greater (49). It is most often used to determine whether or not a particular carbon-hydrogen bond is cleaved in the rate-determining step of a reaction.

It has previously been shown that the hydride transfer steps in the UDPGluDH reaction are not rate-determining (see Section 1.3.6 and reference 24). This conclusion was reached after UDP-(6,6-di-^{2}H)glucose was tested as a substrate for the enzyme. No isotope effect was observed when the rates of reaction using the deuterated versus non-deuterated substrates were compared. In this work, the di-deuterated UDPG was enzymatically synthesized and tested as a substrate for the E141Q, E145Q, and T118A mutants of UDPGluDH. This could be used to determine whether a loss in enzymatic activity as a result of a mutation could be attributed to an effect on a hydride transfer step of the reaction.

3.1.1 An Explanation of Primary Isotope Effects

The deuterium isotope effect is attributed to the difference in the zero-point energies of the stretching vibrations of bonds to hydrogen and deuterium (48). The detailed theoretical explanation of this effect is quite complex, but many of the important aspects of the problem can be greatly simplified to a model that is adequate for most experimental work. The lowest vibrational energy state of a C-H bond lies above the lowest point on the potential energy curve for the system (Figure 3.1). This difference in energy is called the zero-point energy and is equal

to ½$h\nu$, where h is Planck's constant and ν is the frequency of the C-H vibration. The zero-point energy of a C-D bond is lower on the curve than that of a C-H bond, because the stretching frequency of a C-D bond is lower than that of a C-H bond. This is mainly attributed to the larger mass of a deuterium atom.

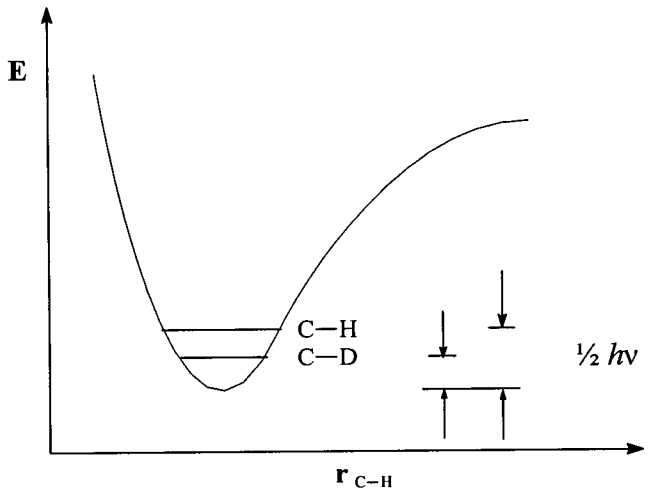


Figure 3.1 The difference in the zero point energies of C-H and C-D bonds (adapted from ref. 48).

If a hydrogen atom is transferred to an acceptor B, the stretching vibration is converted to translational motion as the hydrogen moves toward the acceptor at the highest point of the energy profile (Figure 3.2). Because the C-D and C-H compounds are reaching the same energy maximum but are starting from different energy levels, it will take more energy to bring the C-D compound to the transition state. This causes a reduction in the observed reaction rate when the deuterated compound is used. The calculated maximum for the isotope effect k_H/k_D involving C-H bonds is about 7 at room temperature (49), although much larger effects may be observed if quantum mechanical tunnelling is important in the hydrogen transfer (48).

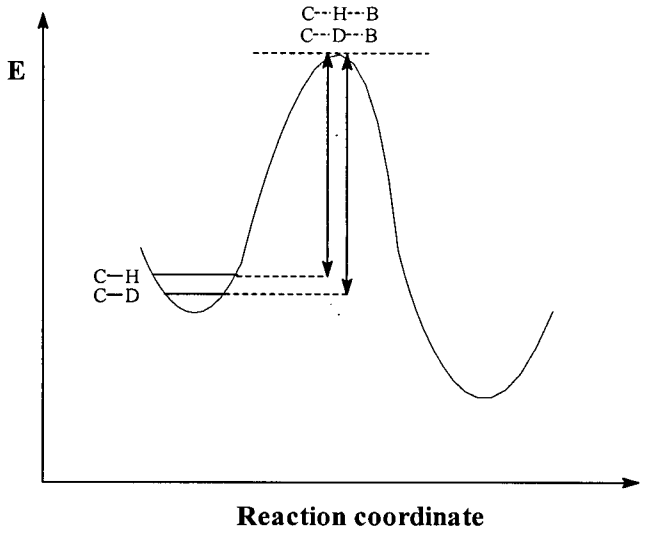


Figure 3.2 A reaction coordiate diagram for a hydrogen (deuterium) transfer reaction in which the zero point energy is lost in the transition state. The difference in the reaction rates of hydrogen and deuterium compounds should correspond to the difference in the zero-point energies of the bonds to these atoms in the ground state (adapted from ref. 48).

3.1.2 Isotope Effects in the UDPGluDH Reaction

Figure 3.3 (a) presents a hypothetical reaction coordinate diagram for the UDPGluDH reaction. Estimates of the relative barriers leading to the mechanistic intermediates are based on previous studies with the wild-type enzyme (Section 1.3). These indicate that the rate-determing step is either substrate binding, hydrolysis of the thioester, or product release. The curves marked with an asterisk (*) indicate the effect of substituting both C-6 hydrogen atoms for deuterium. The energies required for the hydride transfer steps have increased but are still lower than the energy needed for the hydrolysis step. This explains why there was no observed isotope effect for wild-type UDPGluDH.

Figure 3.3 (b) illustrates one possible effect of mutating an important active site residue in the enzyme. If the mutated residue plays a role in the transfer of one (or both) of the hydrogen atoms to NAD^+, then the alteration of this residue may lead to a significant increase in the barrier

of the hydride transfer steps. These barriers may now be greater than those for binding, release, or hydrolysis, and the hydride transfer steps may have become rate-limiting. In this case, substituting both hydrogen atoms for deuterium, indicated with by the curves marked with an asterisk (*), will cause a decrease in the reaction rate that can be measured experimentally. Thus, a kinetic isotope effect would be observed.

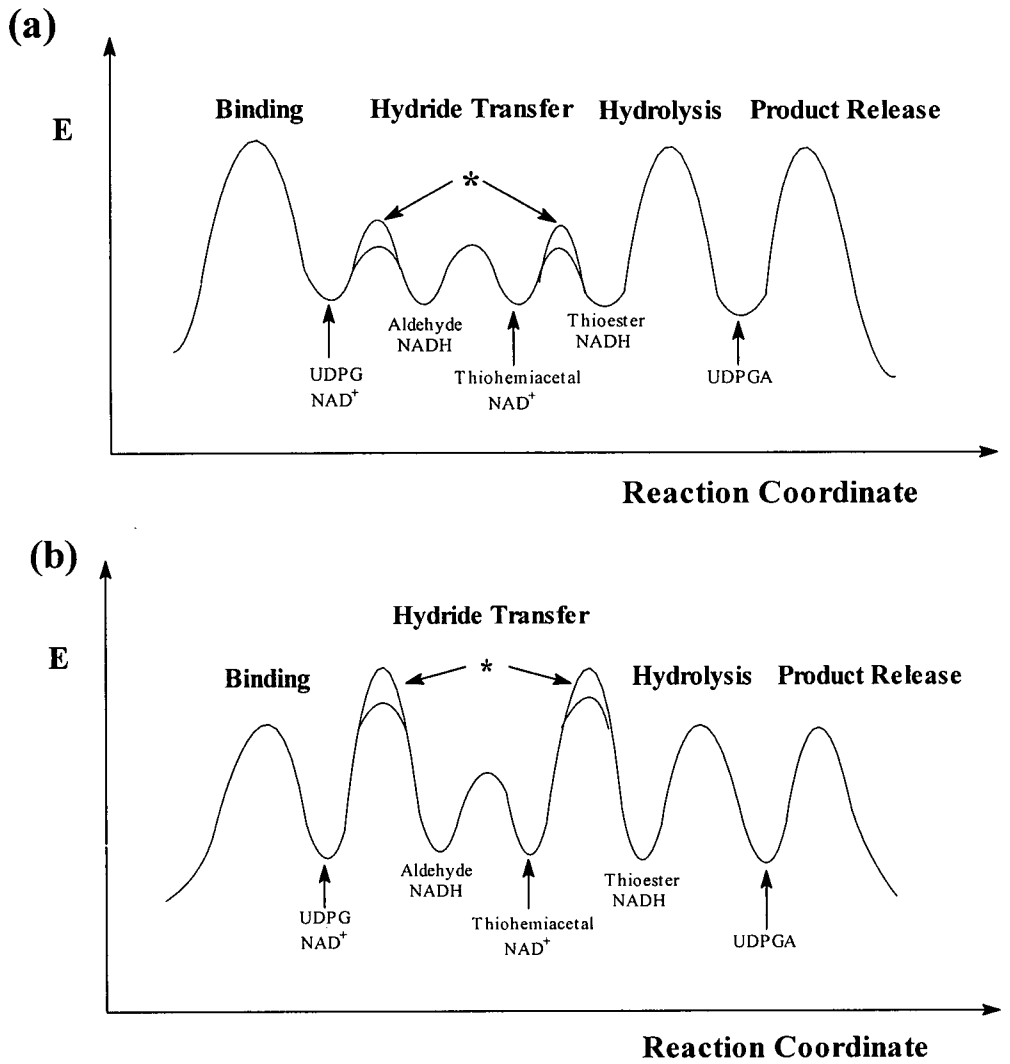


Figure 3.3 **(a)** A reaction coordinate diagram for wild-type UDPGluDH. **(b)** A reaction coordinate diagram for UDPGluDH in which a residue that is important in the hydride transfer steps has been mutated. The relative barriers for binding, hydrolysis, and release are unknown.

3.1.3 Synthesis of UDP-(6,6-di-^{2}H)Glucose

The starting material for this reaction was D-(6,6-di-^{2}H)glucose, which is commercially available. It was enzymatically coupled to a UDP group by the successive action of the enzymes hexokinase, phosphoglucomutase, and UDP-glucose pyrophosphorylase (Figure 3.4). In the first step of this reaction, C-6 of the glucose moiety is phosphorylated by hexokinase. This enzyme catalyzes the transfer of a phosphoryl group from ATP to a variety of six-carbon sugars, and it requires a divalent metal ion for activity. Next, phosphoglucomutase transfers the phosphate group from C-6 to C-1 of glucose, generating the α-anomer of glucose-1-phosphate. The catalytic site of an active mutase contains a phosphorylated serine residue, so the presence of glucose-1,6-diphosphate ensures that the enzyme remains activated. Treatment of glucose-1-phosphate with UDP-glucose pyrophosphorylase and a 10-fold excess of UTP generates the final product. This reaction is readily reversible, but the liberated pyrophosphate (P2) from UTP is rapidly hydrolyzed by inorganic pyrophosphatase. This drives the reaction in the direction of UDP-glucose.

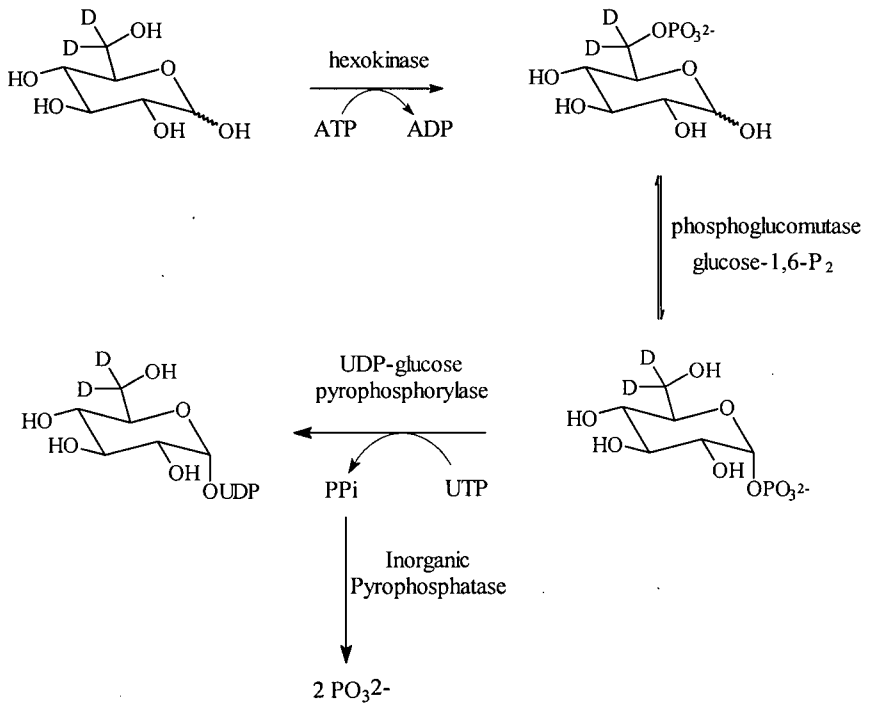


Figure 3.4 Enzymatic coupling of UDP to glucose.

The synthesis of di-deuterated UDP-glucose has been previously described in our laboratory (24). The enzymatic coupling reaction took place in a single incubation step, at 30 °C for 19 hours. The product was purified using a diethylaminoethylcellulose (DE52) ion exchange column that was eluted with a linear gradient of 0 – 400 mM triethylamine bicarbonate buffer. The fractions containing deuterated UDP-glucose were passed through a column of Amberlite IR-120 (plus) resin and then desalted on a Bio-gel P-2 column. Analysis by both ^{1}H NMR (Figure 3.5) and Liquid Secondary Ion mass spectrometry (LSIMS) (Figure 3.6) indicated that the UDPG produced was over 95 % enriched with deuterium at the C-6 position of glucose. The yield of deuterated UDP-glucose was 66 %. This was determined enzymatically by incubating with a known amount of NAD^{+} and a large excess of wild-type enzyme and monitoring the change in absorbance at 340 nm. This assay was used to quantitate all of the solutions used in

the kinetic assays. Small impurities and the presence of counterions or waters of hydration prohibit the use of simple weighing of samples in the determination of accurate UDP-glucose concentrations.

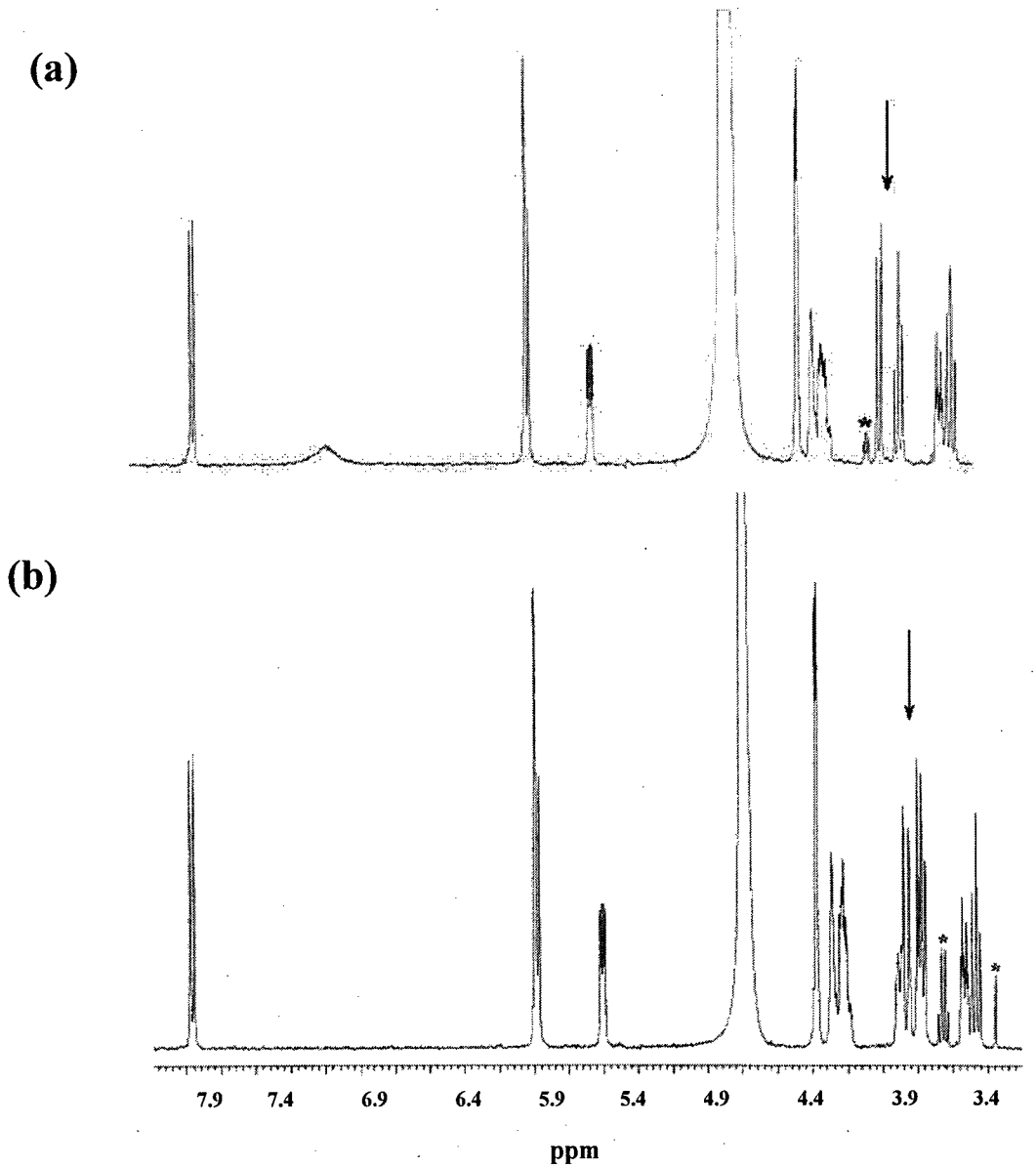


Figure 3.5 ^{1}H NMR spectrum of (a) deuterated and (b) non-deuterated UDPG. In spectrum (b), the signals due to H6'' overlap with those of H3'' (triplet, 3.76 ppm) and H5''(doublet, 3.88 ppm). No such overlap exists in spectrum (a) since the hyrdogen atoms on C6'' are replaced by deuterium. Arrows point to the difference between the two spectra, and impurity peaks are indicated with an asterisk (*).

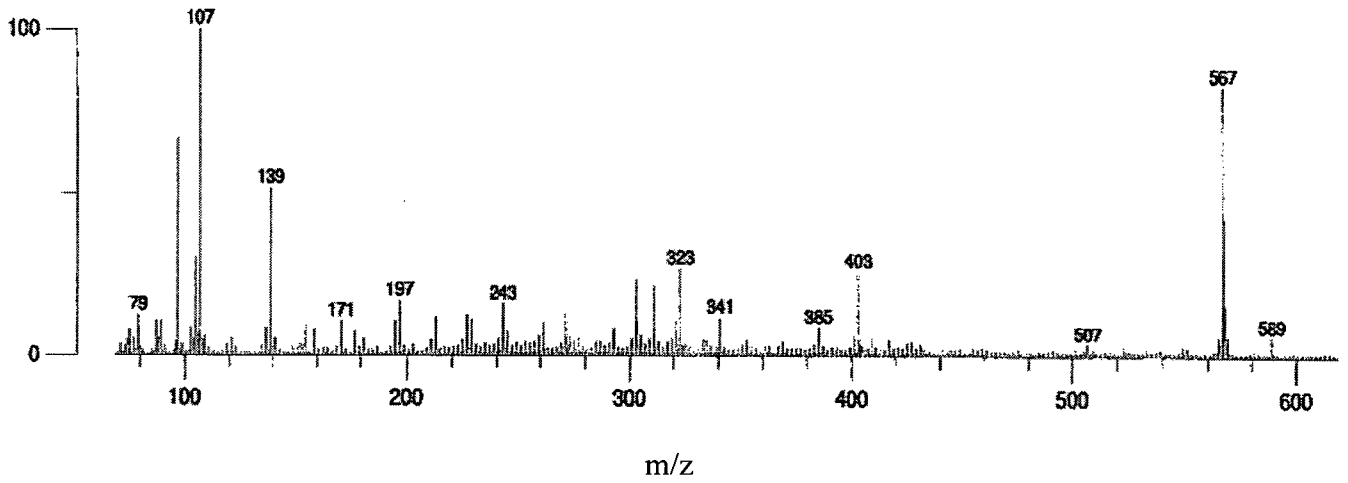


Figure 3.6 Mass spectrum of UDP-(6,6-di-^{2}H)glucose. The peak with *m/z* = 567 corresponds to a compound with the formula $C_{15}H_{21}O_{17}D_2P_2N_2$.

3.2 PRIMARY KINETIC ISOTOPE EFFECT STUDIES

The three mutants of UDPGluDH that were purified to homogeneity, T118A, E141Q, and E145Q, were assayed to determine whether a primary kinetic isotope effect slowed the reaction of UDP-(6,6-di-^{2}H)glucose. Each was tested in the regular UDPGluDH assay, using both labelled and unlabelled UDPG, under saturating conditions of both substrates. The experiment was performed using wild-type UDPGluDH before it was attempted with each mutant protein, to ensure that previous results could be repeated (that is, to show that there was no kinetic isotope effect with the wild-type enzyme). The reported values are averages of three experiments.

3.2.1 Studies Using T118A

The value of k_H/k_D for this mutant was 1.9 ± 0.1, indicating that a C-H bond is being broken in the rate determining step of the reaction. This implies that Thr 118 is more important in the hydride transfer steps than in the hydrolysis step of the UDPGluDH mechanism. In the wild-type enzyme, isotope effects must certainly slow the intrinsic rates of the hydride transfer

steps; however, these effects are masked by rate-determining steps that are not isotopically sensitive (Figure 3.3a). With the mutant enzyme, these isotope effects have been "unmasked", indicating the barriers to hydride transfer have increased significantly with respect to other steps in the reaction.

It was previously determined that the k_{cat} value for T118A UDPGluDH was decreased by a factor of 100 when compared to that of the wild-type enzyme (Section 2.3.3). This would correspond to roughly 2.8 kcal/mol of energy increase in the barrier for the reaction, but does not reveal which of the mechanistic steps are affected. However, since a primary kinetic isotope effect slowed the reaction of UDP-(6,6-di-^{2}H)glucose when incubated with the T118A mutant, one can reasonably assume that the hydride transfer steps are most affected by the mutation.

As discussed in Section 1.4.6, the oxidation of UDPG proceeds with general base catalysis. There are two candidates for the role of general base – Lys 204 and a water molecule that is activated by Asp 264. This water molecule also forms a hydrogen bond to Thr 118; it is likely that this interaction is important for proper positioning of the water. The experimentally determined k_H/k_D value for T118A lends support to the theory that it is the active site water molecule acting as the catalytic base. Mutating Thr 118 likely affected the position of this water, which may cause a significant decrease in the rate of one or both of the hydride transfer steps due to a lack of general base catalysis. The magnitude of the isotope effect was not very large, however, so this conclusion is speculative. It is possible that the water molecule is simply providing electrostatic stabilization and when it is improperly positioned, hydride transfer becomes the rate-limiting step. Further experiments, especially with the K204A mutant, will provide more conclusive evidence as to the identity of the general base involved in the mechanism.

3.2.2. Studies Using E141Q and E145Q

The value of k_H/k_D for E141Q was determined to be 1.45 ± 0.04 and that of E145Q was 1.43 ± 0.05. Only a moderate rate-reduction was observed with both of these mutants, but the isotope effect changes were clearly measurable. Therefore, the main result of these mutations was likely to affect the hydride transfer steps. It is not too surprising that the mutation of residues directly in the active site could raise the barriers to the chemical steps of the enzymatic reaction. A value of ~1.4 for the ratio of k_H/k_D indicates that the hydride transfer steps are not clearly rate-determining in the overall mechanism of the mutant proteins, but they have become partially rate-determining.

3.3. CONCLUSIONS

Many of the details of the mechanism of UDP-glucose dehydrogenase from *Streptococcus pyogenes* have been determined through biochemical investigations within our laboratory. The structure of the enzyme has been elucidated, which has provided a close-up view of the spatial positioning of the important active site residues. Based on this structure, several mutant forms of the enzyme were constructed and these were the focus of the work presented in this thesis. The studies of three of these mutants – K204A, N208A, and D264N – were hindered by inclusion body formation during protein overexpression. The remaining three mutant enzymes – T118A, E141Q, and E145Q – were purified to homogeneity and their kinetic constants were determined. The K_m and k_{cat} values for E141Q and E145Q were within an order of magnitude of the wild-type values, indicating that these residues are not vital to the mechanism of UDPGluDH. However, it appears that Thr 118 is fairly important in the catalytic mechanism because the k_{cat} value of T118A was reduced by a factor of 100 when compared to

the wild-type enzyme. When T118A UDPGluDH was incubated with UDP-(6,6-di-^{2}H)glucose, the value of k_H/k_D was determined to be 1.9 ± 0.1, indicating that a C-H bond is broken in the enzyme's rate-determining step. This differs from previously obtained results with the wild-type enzyme, in which it was found that the hydride transfer steps were not rate-limiting. It appears that the decrease in rate due to the mutation of Thr 118 is largely due to an increase in the energy barrier for the hydride transfer steps of the overall reaction. This supports a catalytic mechanism in which an active site water molecule that is hydrogen-bonded to Thr 118 acts as a catalytic acid/base group.

Future studies on UDPGluDH will initially focus on the three mutant enzymes that were formed as inclusion bodies – K204A, N208A, and D264N. Solubilization of these inclusion bodies, followed by renaturation of the enzymes, may allow large quantities of the mutants to be purified. The mutated residues appear to be involved in stabilizing intermediates in the enzymatic mechanism through the formation of an electrostatically positive pocket in the active site. Determination of the kinetic constants of these mutants may provide an indication of the importance of each residue in the overall mechanism. Further studies on the mutant enzymes using deuterated UDP-glucose, the aldehyde intermediate, and UDC may allow specific roles for the residues to be assigned. Such studies may also provide a basis for other interesting mutations of UDPGluDH.

3.4 MATERIALS AND METHODS

3.4.1 General Procedures

All chemicals were purchased from Sigma, Aldrich, or Fisher Scientific. The separation during the DE52 and Biogel columns was monitored using a Spectra/Chrom Flow Thru UV monitor controller (Spectrum) with a 254 nm filter. ^{1}H Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WH-400 MHz instrument at a field strength of 400 MHz and chemical shifts are reported using the δ scale in ppm. Liquid secondary ion mass spectrometry (LSIMS) was performed with a Kratos Concept II HQ mass spectrometer at the Mass Spectrometry lab of The University of British Columbia. Wild-type UDPGluDH was obtained following a previously published procedure (20) and mutant enzymes were obtained and purified according to the methods outlined in Chapter 2.

3.4.2 Specific Procedures

Synthesis of UDP-(6,6-di-^{2}H)Glucose

The starting material for the enzymatic coupling reaction, D-(6,6-di-^{2}H)glucose, was purchased from Aldrich. The reaction was carried out in an incubation mixture of the following composition: D-(6,6-di-^{2}H)glucose (8 mg, 44 μmol, 1.1 mM), ATP (72 mg, 132 μmol), UTP (72 mg, 132 μmol), glucose-1,6-diphosphate (0.165 μmol), $MgSO_4$ (36 mg, 146 μmol), 70 mM Tris-HCl, pH 7.8 (40 mL), hexokinase (66 units), phosphoglucomutase (109 units), UDP-glucose pyrophosphorylase (12.5 units), and inorganic pyrophosphatase (33 units). The incubation was performed at 30 °C over a period of 19 hours. The reaction mixture was loaded onto a 90 mL DE52 column and eluted with a linear gradient of 0 – 400 mM triethylamine bicarbonate buffer (800 mL total volume). This buffer was prepared by bubbling CO_2 into a 400 mM triethylamine

solution over a period of 12 hours to give a buffer with a pH of 7.4. The eluent fractions were assayed for UDP-glucose by incubating with wild-type UDPGluDH and NAD^+ (0.5 mM) and monitoring the absorbance at 340 nm. The fractions containing deuterated UDP-glucose were pooled and evaporated to dryness using a Savant SC110A Speed Vac Plus. The product was dissolved in water and lyophilized, then redissolved in water and lyophilized again. The resulting solid was dissolved in 6 mL of water and passed through a column of Amberlite IR-120 (plus) resin (10 mL, Na^+ form, eluted with 12 mL of water). This column was prepared by washing the H^+ form of the resin with 1.0 M NaOH until basic to litmus, followed by water until neutral. The collected solution was desalted on a Bio-gel P-2 column (2.5 × 45 cm, eluted with water at a rate of 1 mL/min). The fractions were assayed for UDP-glucose as previously described; those containing the product were pooled and evaporated to dryness to yield a white solid (29 μmol, 66 % yield). The ^1H NMR spectrum agreed with the previously reported results (26). LR LSI(-) MS (thioglycerol matrix) *m/z* = 567, corresponding to the formula $C_{15}H_{21}O_{17}D_2P_2N_2$.

NMR spectroscopic and LSIMS mass spectrometric analyses indicated that the extent of deuterium incorporation was > 95 %.

Enzymatic Determination of the Yield of UDP-(6,6-di-^{2}H)glucose

An assay mixture containing 50 mM Trien-HCl pH 8.7, 2 mM DTT, 200 μg UDPGluDH, and 5 mM NAD^+ was incubated at 30 °C for 5 minutes, or until a stable baseline was obtained at 340 nm. The reaction was then initiated by the addition of a stock solution of known concentration of non-deuterated UDP-glucose, giving a final volume of 1 mL. The enzymatic reaction went to completion and the change in absorbance values was used to calculate the

amount of UDPG added to the mixture. This calculation assumed that 2 equivalents of NADH were produced for every molecule of UDPG consumed, and the extinction coefficient for NADH was 6220 M^{-1}. The difference in the calculated and known values was < 15%. The same procedure was performed using the product from the enzymatic coupling reaction dissolved in 4 mL of water to reveal the yield of deuterated UDPG. For both substrates, the above procedure was repeated twice and an average UDPG concentration was calculated. This method gave a yield of 29 μmol (66 %) for UDP-(6,6-di-^{2}H)glucose.

Primary Kinetic Isotope Effect Experiments

The following procedure was used for T118A UDPGluDH. Slight variations in the substrate and enzyme concentrations were used with E141Q and E145Q; these are indicated in parentheses. The concentrations of both the deuterated and non-deuterated UDPG stock solutions were determined before carrying out each isotope experiment. The rates of oxidation were determined three times for both substrates. The errors reported for the isotope effects represent the standard deviation of the data points from the average determined rates.

The assay mixtures (1 mL final volume) contained 50 mM Trien-HCl pH 8.7, 2 mM DTT, 4 mM NAD^+ (2 mM for both E141Q and E145Q) and 740 μg T118A (100 μg of E141Q and 60 μg of E145Q). These components were mixed and allowed to equilibrate at 30 °C for 6 minutes, or until a stable baseline at 340 nm was observed. The reactions were initiated by the addition of either deuterated or non-deuterated UDP-glucose at a final concentration of 0.5 mM (0.5 mM for E141Q and 0.8 mM for E145Q). The initial velocities were determined from the observed rate of NADH production, as indicated by the absorbance increase at 340 nm. These were then averaged and used to obtain values of k_H/k_D for each mutant.

REFERENCES

1) Feingold, D. S., and Franzen, J. S. (1981) *Trends Biochem. Sci.* **6**, 103-105.

2) De Luca, C., Lansing, M., Crescenzi, F., Martini, I., Shen, G.-J., O'Regan, M., and Wong, C.-H. (1996) *Bioorg. & Med. Chem.* **4**, 131-142.

3) Dalessandro, G., and Northcote, D. H. (1977) *Biochem. J.* **162**, 267-279.

4) Dougherty, B.A., and van de Rijn, I. (1993) *J. Biol. Chem.* **268**, 7118-7124.

5) Arrecubieta, C., Lopez, R., and Garcia, E. (1994) *J. Bacteriol.* **176**, 6375-6383.

6) Moxon, E. R., and Kroll, J. S. (1990) *Curr. Top. Microbiol. Immunol.* **150**, 65-85.

7) Cross, A.S. (1990) *Curr. Top. Microbiol. Immunol.* **150**, 87-95.

8) Strominger, J. L., Kalchar, H. M., Axelrod, J., and Maxwell, E. S. (1954) *J. Am. Chem. Soc.* **76**, 6411.

9) Zalitis, J., and Feingold, D. S. (1969) *Arch. Biochem. Biophys.* **132**, 457-465.

10) Nelsestuen, G. L., and Kirkwood, S. (1971) *J. Biol. Chem.* **246**, 3828-3834.

11) Ridley, W. P., and Kirkwood, S. (1973) *Biochem. Biophys. Res. Commun.* **54**, 955-960.

12) Ridley, W. P., Houchins, J. P., and Kirkwood, S. (1975) *J. Biol. Chem.* **250**, 8761-8767.

13) Ordman, A. B., and Kirkwood, S. (1977) *J. Biol. Chem.* **252**, 1320-1326.

14) Eccleston, E. D., Thayer, M. L., and Kirkwood, S. (1979) *J. Biol. Chem.* **254**, 11399-11404.

15) Schiller, J. G., Bowser, A. M., and Feingold, D. S. (1972) *Carbohyd. Res.* **25**, 403-410.

16) Schiller, J. G., Bowser, A. M., and Feingold, D. S. (1973) *Biochim. Biophys. Acta.* **293**, 1-10.

17) Schiller, J. G., Lamy, F., Frazier, R., and Feingold, D. S. (1976) *Biochim. Biophys. Acta.* **453**, 418-425.

18) Arrecubieta, C., Garcia, E., and Lopez, R. (1996) *J. Bacteriol.* **178**, 2971-2974.

19) Dillard, J. P., Vandersea, M. W., and Yother, J. (1995) *J. Exp. Med.* **181**, 973-983.

20) Campbell, R. E., Sala, R. F., van de Rijn, I., and Tanner, M. E. (1997) *J. Biol. Chem.* **272**, 3416-3422.

21) Campbell, R. E., Mosimann, S. C., van de Rijn, I., Tanner, M. E., and Strynadka, N. C. J. (2000) *Biochemistry* **39**, 7012-7023.

22) Ordman, A. B., and Kirkwood, S. (1977) *Biochim. Biophys. Acta.* **481**, 25-32.

23) Campbell, R. E., and Tanner, M. E. (1999) *J. Org. Chem.* **64**, 9487-9492.

24) Ge, X. (2000) *Covalent Catalysis in the UDP-Glucose Dehydrogenase Reaction.* Ph.D. thesis, The University of British Columbia.

25) Campbell, R. E., and Tanner, M. E. (1997) *Angew. Chem. Int. Ed. Engl.* **13/14**, 1520-1522.

26) Ge, X., Campbell, R. E., van de Rijn, I., and Tanner, M. E. (1998) *J. Am. Chem. Soc.* **120**, 6613-6614.

27) Campbell, R. E. (2000) *The Structure and Mechanism of UDP-Glucose Dehydrogenase.* Ph.D. thesis, The University of British Columbia.

28) Barycki, J. J., O'Brien, L. K., Bratt, J. M., Zhang, R., Sanishvili, R., Strauss, A. W., and Banaszak, L. J. (1999) *Biochemistry* **38**, 5786-5798.

29) Adams, M. J., Ellis, G. H., Gover, S., Naylor, C. E., and Phillips, C. (1994) *Structure* **2**, 651-668.

30) Zhang, L., Chooback, L., and Cook, P. F. (1999) *Biochemistry* **38**, 11231-11238.

31) Dao-pin, S., Anderson, D. E., Baase, W. A., Dahlquist, F. W., and Matthews, B. W. (1991) *Biochemistry* **30**, 11521-11529.

32) Neidhart, D. J., Howell, P. L., and Petsko, G. A. (1991) *Biochemistry* **30**, 9264-9273.

33) Drlica, K. (1997) in *Understanding DNA and Gene Cloning: A Guide for the Curious*, 3rd Ed., John Wiley & Sons, Inc., New York, NY.

34) Stryer, L. (1995) in *Biochemistry*, 4th Ed., W. H. Freeman and Company, New York, NY.

35) Franzen, J. S., Ishman, R., and Feingold, D. S. (1976) *Biochemistry* **15**, 5665-5671.

36) Grubmeyer, C. T., and Gray, W. R. (1986) Biochemistry **25**, 4778.

37) Teng, H., Segura, E., and Grubmeyer, C. (1993) *J. Biol. Chem.* **268**, 14182-14188.

38) Nagai, A., Kheirolomoom, A., and Ohta, D. (1993) *J. Biochem.* **114**, 856.

39) Patra, A. K., Mukhopadhyay, R., Mulhija, R., Krishnan, A., Garg. L. C., and Panda, A. K. (2000) *Protein Expression and Purification* **18**, 182 – 192.

40) Yang, Q.-H, Wu, C.-L, Lin, K., and Li, L. (1997) *Protein Expression and Purification* **10**, 320 – 324.

41) Misawa, S., and Kumagai, I. (1999) *Biopolymers (Peptide Science)* **51**, 297 – 307.

42) Snyder, S. W., Edalji, R. P., Lindh, F. G., Walter, K. A., Solomon, L., Pratt, S., Steffy, K., Holtzman, T. F. (1996) *Journal of Protein Chemistry* **15**, 763-774.

43) Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104 – 137.

44) Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 173 – 187.

45) Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 188 – 196.

46) Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248 – 254.

47) Laemmeli, U. K. (1970) *Nature* **227**, 680 – 685.

48) Jencks, W. P. (1969) in *Catalysis in Chemistry and Enzymology*, Dover Publications Inc., New York, NY.

49) Carey, F. A., and Sundberg, R. J. (1990) in *Advanced Organic Chemistry Part A: Structure and Mechanism*, 3rd Ed, Plenum Press, New York, NY.