STRUCTURAL CHARACTERIZATION OF ESSENTIAL ENZYMES INVOLVING SIALIC ACID METABOLISM IN BACTERIAL PATHOGENS

by

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ABSTRACT

Sialic acid plays vital roles in various biological processes including cellular recognition and cell adhesion. The biosynthesis and post-synthetic processing of sialic acid is particularly important to host-pathogen interactions because many virulent bacteria decorate their cell surfaces with sialic acid-containing molecules in order to evade the host's immune response.

Neisseria meningitidis, a highly invasive human pathogen that causes bacterial meningitis, produces a capsular polysaccharide comprised of polysialic acids that protect the bacteria from the host's immune system by mimicking the sialic acid-containing cell surface structures. The biosynthesis of sialic acid is catalyzed by sialic acid synthase NeuB. We report the structural and biochemical analysis of the first potent inhibitor of sialic acid synthase from *N. meningitidis*. The inhibitor was synthesized as a mixture of stereoisomers, which mimics the tetrahedral intermediate of the NeuB reaction. Based on the crystallographic and kinetic analysis of the inhibitor binding, an improved mechanism is proposed.

Capsular polysaccharides of certain strains of *N. meningitidis* can be further acetylated by sialic acid-specific *O*-acetyltransferases, a modification that correlates with the virulence in bacterial infection. In the second part of the thesis, we report the first kinetic and structural analysis of bacterial sialic acid *O*-acetyltransferase OatWY from *N. meningitidis*. Crystals of OatWY were obtained in complex with either CoA, acetyl-CoA, or nonhydrolyzable donor analogue *S*-(2-oxopropyl)-CoA. Structural analysis in combination with kinetic and mutagenesis studies elucidates the mechanistic features and substrate specificity of this enzyme.

Campylobacter jejuni, a leading causative agent of bacterial diarrhea and gastroenteritis, expresses sialylated lipooligosaccharide, which mimics the carbohydrate structure of human gangliosides. Sialyltransferase Cst-II is the enzyme responsible for the lipooligosaccharide sialylation in *C. jejuni* as a means of evading the host's immune system. The last part of the thesis describes the first ternary complex of Cst-II with the donor analogue CMP and the terminal trisaccharide (Neu5Ac- α -2,3-Gal- β -1,3-GalNAc) of its natural acceptor. Site-directed mutagenesis of acceptor binding residues was performed and mutants were characterized by enzyme kinetics. Our results reveal the structural basis for the binding of a physiologically relevant natural acceptor and provide additional insight into the mechanism and acceptor specificity of this enzyme.

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ABBREVIATIONS

aa	amino acid(s)	
AcCoA	acetyl coenzyme A	
Acetyl-CoA	acetyl coenzyme A	
ACN	acrylonitrile	
ABC	ATP-binding cassette	
ADSC	Area Detection Systems Corporation	
ALS	Advanced Light Source	
APBS	Adaptive Poisson-Boltzmann Solver	
АТР	adenosine 5'-triphosphate	
BSA	bovine serum albumin	
CAZy	Carbohydrate-Active enzymes	
CCD	charge-coupled device	
CDP	cytidine 5'-diphosphate	
СМР	cytidine 5'-monophosphate	
CMP-3FNeu5Ac	CMP-3-fluoro- <i>N</i> -acetyl-neuranminic acid	
CoA	coenzyme A	
СРК	Corey, Pauling, Kultin	
СТР	cytidine 5'-triphosphate	
DMAP	4-Dimethylaminopyridine	
DNA	deoxyribonucleic acid	
DTT	dithiothreitol	

EDTA	ethylenediaminetetraacetic acid		
ESI-MS	electrospray ionization mass spectrometry		
Gal	galactose		
GAT	galactoside acetyltransferase		
GalNAc	N-acetyl-galactosamine		
Glc	glucose		
GlcNAc	N-acetyl-glucosamine		
GT	glycosyltransferase		
GT-A	glycosyltransferase A		
GT-B	glycosyltransferase B		
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid		
HPLC	high pressure liquid chromatography		
HRMS	high resolution mass spectrometry		
Hz	Hertz		
IPTG	isopropyl β-D-thiogalactopyranoside		
Kb	kilobase		
k _{cat}	catalytic constant		
K_m	Michaelis constant		
kDa	kilodalton		
KDN	2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid		
KDO	2-keto-3-deoxy-D-manno-octulosonic acid		
LβH	left-handed β-helix		

LDH	lactate dehydrogenase	
LOS	lipooligosaccharide	
MAT	maltose acetyltransferase	
MAD	multi-wavelength anomalous dispersion	
ManNAc	N-acetylmannosamine	
MES	4-morpholineethanesulfonic acid	
MHz	megahertz	
MPD	2-methyl-2,4-pentanediol	
NADH	nicotinamide adenine dinucleotide (reduced form)	
NCAM	neural cell adhesion molecule	
Neu5Ac	N-acetylneuraminic acid	
NeuNAc	N-acetylneuraminic acid	
NMPK	nucleoside monophosphate kinase	
NMR	nuclear magnetic resonance	
OD ₆₀₀	optical density at 600 nm	
PCR	polymerase chain reaction	
PDB	protein data bank	
PEG	polyethylene glycol	
r.m.s.	root mean square	
rpm	revolutions per minute	
PEP	phosphoenolpyruvate	
РК	pyruvate kinase	

S-CoA	S-(2-oxopropyl)-CoA	
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis	
SeMet	selenomethionine	
Sia	sialic acid	
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol	
TLC	thin layer chromatography	
UDP	uridine 5'-diphosphate	
V _{max}	maximum rate	
WT	wild type	
v/v	unit volume (ml) per unit volume (ml)	
w/v	unit weight (g) per unit volume (ml)	

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DEDICATION

To my parents

CO-AUTHORSHIP STATEMENT

Chapter 2 contains a version of manuscript published in *Biochemistry* [Liu, F., Lee, H.J., Strynadka, N.C.J., and Tanner, M.E. (2009) Inhibition of *Neisseria meningitidis* Sialic Acid Synthase by a Tetrahedral Intermediate Analogue. **Biochemistry** 48: 9194-9201.] I performed protein expression and purification, crystallization, crystal-inhibitor soaking, data collection, structure determination, and refinement. Design and synthesis of the inhibitor followed by inhibition kinetics were performed by Feng Liu. The manuscript were prepared by myself, Feng Liu, and Dr. Martin Tanner, and revised by Drs. Marin Tanner and Natalie Strynadka. All structural figures and crystallographic table were prepared by myself.

Chapter 3 a version of manuscript published in *Journal of Biological Chemistry* [Lee, H.J., Rakić, B., Gilbert, M., Wakarchuk, W.W., Withers, S.G., and Strynadka, N.C.J. (2009) Structural and Kinetic Characterizations of the Polysialic Acid *O*-Acetyltransferase OatWY from *Neisseria meningitidis*. **Journal of Biological Chemistry** 284: 24501-24511.] Dr. Michel Gilbert provided the genomic DNA of the *N. meningitidis* serogroup Y. Dr. Warren Wakarchuk provided the sample of *N. meningitidis* serogroup Y polysaccharide. I designed a construct and performed molecular cloning, protein expression and purification, site directed mutagenesis for active site mutants, and kinetic analysis. Crystallization, substrate soaking, data collection, structure determination, refinement, and molecular modeling were all performed myself. Deacetylation of the polysaccharide sample, preparation of the disaccharide compound (Glc-(α 1 \rightarrow 4)-Sia) and *S*-(2-oxopropyl)-CoA were performed by Dr. Bojana Rakić. I prepared all manuscript text and figures, except for the text of the preparation of saccharide samples and *S*-(2-oxopropyl)-CoA in the experimental procedures of the manuscript. This portion of the text was prepared by Dr. Bojana Rakić. All figures and tables were prepared by myself. Drs. Natalie Strynadka, Stephen Withers, and Warren Wakarchuk revised the manuscript.

For Chapter 4, I performed protein expression and purification, site-directed mutagenesis, crystallization, substrate soaking, data collection, structure determination, refinement, and molecular modeling. Dr. Jamie Rich performed the synthesis of the acceptor substrates and kinetic analysis of active site mutants. I prepared all manuscript text and figures, except for the text of the synthesis of acceptor compounds and enzyme kinetics in the experimental procedures of the manuscript. This portion of the text was prepared by Dr. Jamie Rich. Dr. Warren Wakarchuk provided the initial full length construct of Cst-II. All structural figures and crystallographic table were prepared by myself. Table for kinetic analysis was prepared by Dr. Jamie Rich. Drs. Natalie Strynadka revised the manuscript.

<u>CHAPTER 1</u>: INTRODUCTION

1.1 Sialic acid

Sialic acids are a large family of negatively charged nine-carbon monosaccharides comprising over 50 known derivatives, which share a common core structure of neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranos-1-onic acid, NeuNAc) (Figure 1.1) (Varki, 1992, Schauer, 2000, Angata & Varki, 2002, Vimr & Lichtensteiger, 2002).



Figure 1.1: Structures of sialic acids.

(A) Neuraminic acid (Neu, R = H), *N*-acetylneuraminic acid (Neu5Ac, R = CH₃CO–) and *N*-glycolylneuraminic acid (Neu5Gc, R = HOCH₂CO–). (B) 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (KDN). (C) 5,7-diamino-3,5,7,9-tetradeoxy-D-glycero-D-galacto-nonulosonic acid (legionaminic acid) derivative. (D) 5,7-diamino-3,5,7,9-tetradeoxy-L-glycero-L-manno-nonulosonic acid (pseudaminic acid) derivative. (E) 2-keto-3-deoxy-D-manno-octulosonic acid (KDO).

Sialic acids are typically found as the terminal residues attached to the outermost cell surface glycoconjugates in various organisms including mammals, viruses, and bacteria. Sialic acids show a variety of natural modifications including acetylation, and glycolylation (Traving & Schauer, 1998, Angata & Varki, 2002, Schauer, 2009). The reason for this chemical diversity is not entirely clear, however, a reasonable hypothesis is that the modifications on sialic acids are consequences of the evolutionary selection by host-pathogen interactions (Angata & Varki, 2002). In addition to chemical variations within the sialic acid residues, additional diversity arises from various glycosidic linkages (α -2,3-, α -2,6-, and α -2,8-) between sialic acids and the underlying glycans (Table 1.1) (Troy, 1992, Angata & Varki, 2002, Harduin-Lepers *et al.*, 2005). Furthermore, sialic acids contribute to the structural diversity of cell surface glycans due to a variety of potential linkages to the underlying carbohydrate chain of these glycans, and various modifications on sialic acid residues (Schauer, 2000, Schauer, 2004, Varki & Varki, 2007).

1.1.1 Biological Importance of Sialic Acid

Due to their terminal localization and exposure to the extracellular environment at cell surfaces, sialic acids play pivotal roles in various physiological and pathological processes as intrinsic or extrinsic receptors, including masking of cellular recognition sites and mediating cellular interactions, and are closely associated with tumor metastasis (Figure 1.2) (Varki, 1993, Traving & Schauer, 1998, Dall'Olio & Chiricolo, 2001, Angata & Varki, 2002, Hakomori, 2002, Varki & Varki, 2007).

Organism	Reported structure
Bacteria	
Escherichia coli K1	$(-8-\text{Neu5Ac}-\alpha 2-)_n$
Escherichia coli K92	Alternating (-8-Neu5Ac- α 2-) and
	$(-9-\text{Neu5Ac}-\alpha 2-)$ linkages
Neisseria meningitidis B	$(-8-\text{Neu5Ac}-\alpha 2-)_n$
Neisseria meningitidis C	$(-9-\text{Neu5Ac}-\alpha 2-)_n$
Neisseria meningitidis Y	$(-6Glc-\alpha 1, 4-Neu5Ac-\alpha 2-)_n$
Neisseria meningitidis W-135	$(-6Gal-\alpha 1, 4-Neu5Ac-\alpha 2-)_n$
Neisseria meningitidis	Neu5Ac-α2,3-Gal; Neu5Ac-α2,6-Gal
Neisseria gonorrhoeae	Neu5Ac-α2,3-Gal
Haemophilus influenzae	Neu5Ac-α2,3-Gal; Neu5Ac-α2,8-Neu5Ac
Campylobacter jejuni	Neu5Ac-α2,3-Gal; Neu5Ac-α2,8-Neu5Ac
Pasteurella multocida	Neu5Ac-α2,3-Gal; Neu5Ac-α2,6-Gal
Pasteurella haemolytica A2	$(-8-\text{Neu5Ac}-\alpha 2-)_n$
Photobacterium phosphoreum	Neu5Ac-α2,3-Gal
Photobacterium profundum	Neu5Ac-α2,6-Gal
Moraxella nonliquefaciens	$(-8-\text{Neu5Ac}-\alpha 2-)_n$
Vibrio sp.	Neu5Ac-a2,3-Gal
Human	
Ganglioside	Neu5Ac-α2 3-Gal· Neu5Ac-α2 6-Gal·
	Neu5Ac-a2.8-Neu5Ac
Neural cell adhesion molecule	$(-8-\text{Neu}5\text{Ac}-\alpha 2-)_n$
Tumor	$(-8-\text{Neu5Ac}-\alpha 2-)_n$; $(-9-\text{Neu5Ac}-\alpha 2-)_2$
	· · · · · · · · · · · · · · · · · · ·

Table 1.1: Summary of the structure of sialic acid-containing glycoconjugates in bacteria and human.

They are involved in the shielding of recognition sites in macromolecules to protect them from phagocytic degradation or the innate immune response (Kazatchkine *et al.*, 1979, Muller *et al.*, 1983, Kelm & Schauer, 1997, Ram *et al.*, 1998, Ley, 2003). Sialic acids make cells as "self", thereby protecting them from immunological attacks (Schauer, 2004). In contrast, foreign "non-self" cells, which do not display sialic acids on their surfaces, are

prone to degradation by the host immune defense system. For example, complement factor H, a major regulatory protein of the human immune system, binds to sialic acids of cell surface glycoconjugates and protects host cells from the killing cascade of the alternate complement pathway upon the onset of the immune response (Kazatchkine *et al.*, 1979, Pangburn, 2000).



Figure 1.2: Biological roles of sialic acids.

Given their terminal locations and ubiquitous distribution, sialic acids are involved in various physiological and pathological processes.

Sialic acids are important molecules in pathogenesis as they are the most frequently found monosaccharides responsible for microbial infections. Various bacterial and viral pathogens bind to the host cells via sialic acid-mediated interactions (Suzuki *et al.*, 2000, Lehmann *et al.*, 2006, Yoneyama *et al.*, 2008, Haselhorst *et al.*, 2009). These foreign pathogens express sialic acid-recognizing proteins on their surfaces to facilitate attachments to the host cells (Table 1.2) (Angata & Varki, 2002, Varki, 2008). The most well-studied examples of sialic acid-recognizing proteins are hemagglutinins of influenza viruses, which preferentially recognize α -2,3 or α -2,6-linked sialic acids (Suzuki *et al.*, 2000, Auewarakul *et al.*, 2007, Pekosz *et al.*, 2009). Several bacterial pathogens also utilize sialic acids not only to interact with the host cells but also to evade the host's immune response (Ono *et al.*, 1989, Moxon & Kroll, 1990, Preston *et al.*, 1996, Miller-Podraza *et al.*, 1997).

Table 1.2: Example of the pathogens that express sialic acid-recognizing proteins.

Pathogen	<i>Sia</i> -recognizing protein ^a	Target sialylated structure	
Human <i>Influenza</i> A	Hemagglutinin	Sia-α2,6-Gal(NAc)	
Avian <i>Influenza</i> A	Hemagglutinin	Sia-α2,3-Gal	
Human Influenza C Hemagglutinin-esterase		9-O-Ac-Sia	
Vibrio cholerae	Toxin	Gal-β1,3-GalNAc-β1,4-(Sia-α2,3)-Lac-Cer	
Plasmodium falciparum	EBA-175	Sia-α2,3-Gal-β1,3-(Sia-α2,6)-GalNAc	
Clostridium botulinum	Toxin	Polysialogangliosides	
Helicobacter pylori	SabA	Sia-α2,3-Gal on gangliosides	

^{*a*} Sia indicates the sialic acid

1.1.2 Sialylation in Pathogenic Bacteria

Multiple bacterial lipooligosaccharides and capsular polysaccharides are known to contain sialic acids either as terminal residues or in the form of polysialic acids. Although most bacteria do not express sialic acids, certain types of pathogenic bacteria such as *Escherichia coli*, *Neisseria meningitidis*, and *Campylobacter jejuni* can synthesize and express sialic acids on lipopolysaccharide (LPS), in the form of lipooligosaccharide (LOS) and/or capsular polysaccharide (CPS) at their outermost cell surfaces (Moxon & Kroll, 1990, Troy, 1992). The sialic acid-containing capsule of pathogenic bacteria is considered a major virulence factor, which confers resistance to both phagocytosis and the alternate complement pathway of the host's immune system (Horwitz & Silverstein, 1980, Jarvis & Vedros, 1987, Vogel *et al.*, 1996, Bacon *et al.*, 2001).

The capsular polysaccharide (CPS) is the major component of the outer membrane of the cell envelope of the pathogenic bacteria that includes lipid A, core oligosaccharide, and exopolysaccharide (O-antigen) (Figure 1.3) (Raetz & Whitfield, 2002, Nikaido, 2003). Lipid A, the hydrophobic core of LOS and CPS, is a glucosamine-based phospholipid that makes up the outer monolayer of the outer membranes in Gram-negative pathogenic bacteria (Figure 1.4) (Raetz, 1990).



Figure 1.3: Model of the inner and outer membranes of the pathogenic bacteria.

The core oligosaccharide is composed of various sugar components which are directly attached to lipid A. The outer region of the core oligosaccharide provides the attachment site for the polysaccharide (O-antigen), which shows remarkable structural diversity and plays pivotal roles in bacterial pathogenesis (Raetz & Whitfield, 2002, Guo *et al.*, 2008). Pathogenic bacteria commonly produce a thick layer of polysaccharide, which masks various potential antigenic proteins on the bacterial surface that would otherwise provoke the host's immune response and thereby lead to the destruction of the bacteria.

Capsular polysaccharides of pathogenic bacteria are hydrophilic and negatively charged, facilitating the evasion from the attack of phagocytotic cells due to the mutual charge repulsion between polysaccharides and phagocytotic cells (Horwitz & Silverstein, 1980). Pathogenic bacteria also developed resistance to the non-specific host immune defense mechanism through the sialic acid residues of the polysaccharides by inhibiting the alternate complement pathway and the complement-mediated opsonophagocytosis (Jarvis & Vedros, 1987, Cross, 1990). Alternate complement pathway is a natural defense mechanism of the host, which is composed of the activation of various serum proteins in the complement cascade, resulting in the elimination of bacterial pathogens through phagocytosis or cell lysis (Figure 1.4) (Morgan *et al.*, 2005, Schneider *et al.*, 2007).



Figure 1.4: Alternate complement pathway.

The alternate complement pathway leads to the formation of the C3 convertase that cleaves C3 into C3b. Increased cleavage of C3 results in the assembly of C5 convertase, contributing the formation of the membrane attack complex (MAC), and eventually triggers bacterial cell lysis.

The deposition of the opsonic fragment C3b to the bacterial surface is the central process of this immunological pathway. Attachment of C3b to the bacterial surface leads to the activation of a series of serum proteins in the cascade pathway, and eventually leads to the formation of the membrane attack complex (MAC), which attaches to the bacteria for membrane destruction followed by the cell lysis (Winkelstein, 1981, Frank *et al.*, 1987). Furthermore, C3b also mediates the uptake process by phagocytotic cells to eliminate the bacterial pathogens (Ehlenberger & Nussenzweig, 1977, Schneider *et al.*, 2007).

Capsular polysaccharides confer resistance to the complement-mediated opsonophagocytosis by steric effects in which the capsule prevents the deposition of C3b on the surface of the bacteria and simultaneously inhibits the binding of C3b receptors on the phagocytotic cell surface. Furthermore, sialic acids of the capsular polysaccharides bind to the factor H, a major regulatory protein of the alternate complement pathway, which interacts with C3b and degrades it to the inactive form (iC3b), thereby breaking the amplification loop of this immunological pathway (Sim *et al.*, 1981, Moxon & Kroll, 1990).

The sialic acid moiety of the lipooligosaccharide on the outer membrane of the pathogenic bacteria has been shown to inhibit the deposition of C3b on the bacterial surface, thereby blocking the cascade of the alternate complement pathway (Frank *et al.*, 1987). In addition, the capsular polysaccharide functions as a barrier which prevents the binding of complement proteins including the membrane attack complex (MAC). Studies on unencapsulated mutants of pathogenic bacteria, but with partially sialylated lipooligosaccharides, shows similar level of C3b deposition on the surface compared to that of the wild-type strain. However, the unencapsulated bacteria are susceptible to the

alternate complement response due to the lack of the hindrance of MAC insertion by capsules, indicating that capsular polysaccharides protect pathogenic bacteria by inhibiting the proper insertion of MAC into the bacterial membrane (Vogel *et al.*, 1997, Vogel & Frosch, 1999).

In addition to the inhibition of the complement activation and phagocytosis, bacterial polysaccharide capsules are poor immunogens due to their structural similarities to glycoconjugates expressed in the host. The neuroinvasive organisms, *Escherichia coli* K1 and *Neisseria meningitidis* serogroup B, express capsular polysaccharides composed of a homopolymer of α -2,8-linked sialic acids, which mimic the structurally identical polysialic acids on the neural cell adhesion molecule (NCAM) in humans (Troy, 1992, Rutishauser, 2008). Another human pathogen, *Campylobacter jejuni*, displays the terminal sialic acid residues on the capsule of the lipooligosaccharide, and mimics the terminal carbohydrate structures of the sialylated human gangliosides (Guerry *et al.*, 2002). Due to the similarities between bacterial capsules and sialylated glycoconjugates in humans, the bacterial capsules are considered the major virulence determinants to evade the host immune system.

1.2 Genetic Organization of the Gene Cluster Encoding Capsular Polysaccharide Expression

Most extensively studied bacterial pathogens that express capsular polysaccharides are *E. coli* K1 and *N.meningitidis* serogroup B. Both bacterial pathogens contain the gene clusters involved in the biosynthesis and transport of polysaccharides (Silver *et al.*, 1981, Frosch *et al.*, 1989). Although, these pathogenic bacteria express chemically and immunologically identical capsular polysaccharides composed of α -2,8-linked polysialic acids, their capsular polysaccharide gene clusters show slightly different organization, particularly in genes responsible for the polymer transport (Figure 1.5) (Bliss & Silver, 1996).

A. Genetic organization of	the capsular polysialic acid gene c	luster in <i>E. coli</i> I	K 1
├─── Region 3───┤	Region 2		— Region 1 ———
kpsM kpsT neuD neu	B neuA neuC neuE neuS	kpsS kpsC	kpsU kpsD kpsE kpsF
Proposed Functions			
KpsM - ABC-Transporter KpsT - ATPase NeuD - Acetyltransferase NeuB - NeuNAc synthase NeuA - CMP-NeuNAc synthethase NeuC - UDP-GIcNAc 2-epimerase NeuE - Unknown NeuS - Polysialyltransferase	KpsS - Unknown KpsC - Unknown KpsU - CMP-KDO synthetase KpsD - Polysaccharide transport KpsE - Polysaccharide transport KpsF - Arabinose 5-phosphate isomerase		
B. Genetic organization of	the capsular polysialic acid gene c	luster in <i>N. men</i> l	ingitidis B
Region C		Region B	
ctrD ctrC ctrB ct	rA siaA siaB siaC siaD		DA
Proposed Functions			
CtrD - ABC-Transporter CtrC - ABC-Transporter	SiaA - UDP-GIcNAc 2-epimerase SiaB - CMP-NeuNAc synthetase		

Figure 1.5: Genetic organization of the capsular polysaccharide gene clusters in pathogenic bacteria.

SiaC - NeuNAc synthase

SiaD - Polysialyltransferase

CtrB - Polysaccharide transport

CtrA - Polysaccharide transport

LipB - Unknown LipA - Unknown

1.2.1 Genetic Organization of the Capsular Polysaccharide Gene Cluster of E. coli K1

Genetic and molecular analyses resulted in the identification of the 17 kb gene cluster, which encodes the necessary proteins for the synthesis and transport of polysaccharides in E. *coli* K1 (Silver *et al.*, 1981, Vimr *et al.*, 1995). The cluster is divided into three functional regions (Figure 1.5A) (Vimr et al., 1989, Boulnois & Roberts, 1990). The central biosynthetic region (region 2) contains the genes encoding sialic acid synthesis, activation, and polymerization, and is a unique genetic region for each distinct serotype among E. coli (Roberts et al., 1986, Boulnois & Jann, 1989). The flanking regions 1 and 3 encode the genes responsible for polysaccharide export to the bacterial surface and are strongly conserved among serologically distinct capsular polysaccharide gene clusters of E. coli, suggesting the universal mechanism of the polysaccharide export in pathogenic bacteria (Roberts et al., 1988, Vimr et al., 1989, Vimr et al., 1995). Mutational analyses revealed that cells harboring mutations in region 2 do not synthesize detectable levels of polysaccharides, and cells harboring mutations in region 1 or 3 accumulate unexported intracellular polysaccharides (Vimr et al., 1989, Pavelka et al., 1994, Wunder et al., 1994, Vimr *et al.*, 1995).

1.2.2 Genetic Organization of the Capsular Polysaccharide Gene Cluster of N. *meningitidis*

Capsular polysaccharide expression depends on the 24 kb gene cluster, encoding proteins responsible for the biosynthesis and transport of polysaccharides in *N. meningitidis*. This gene cluster consists of three functional regions (region A, B, and C) involved in the

expression and transport of the capsular polysaccharide (Figure 1.5B) (Frosch *et al.*, 1989, Schoen *et al.*, 2009). The genes in region A are involved in the biosynthesis of polysaccharides determining the distinct chemical compositions of the capsules expressed in different meningococcal serogroups including B, C, W-135, or Y (Claus *et al.*, 1997). Both regions B and C are involved in the translocation of the synthesized polysaccharide as revealed by mutational analyses on selected genes in those regions (Frosch *et al.*, 1989, Frosch *et al.*, 1991, Frosch & Muller, 1993).

1.3 Bioisynthesis and Activation of Sialic Acids in Pathogenic Bacteria

The biosynthesis and activation of sialic acids in pathogenic bacteria involves a series of catalytic reactions governed by three distinctive enzymes, UDP-GlcNAc 2-epimerase, sialic acid synthase, and CMP-NeuNAc synthetase (Figure 1.6) (Masson & Holbein, 1983). First, the UDP-GlcNAc 2-epimerase converts UDP-*N*-acetylglucosamine (UDP-GlcNAc) to N-acetylmannosamine (ManNAc) (Murkin et al., 2004, Vann et al., 2004). Secondly, the sialic condensation acid synthase catalyzes the reaction of ManNAc and phosphoenolpyruvate (PEP) to synthesize N-acetylneuraminic acid (NeuNAc, sialic acid) (Vann et al., 1997). Genetic analysis has revealed that the genes encoding these enzymes are indispensible for the biosynthesis of the sialic acid in pathogenic bacteria (Silver *et al.*, 1984, Vimr et al., 1989, Zapata et al., 1992). Finally, the synthesized sialic acid is converted to the active form, CMP-NeuNAc, by CMP-NeuNAc synthetase, which transfers cytidine-5'-monophosphate (CMP) to the sialic acid (Vann et al., 1987, Ganguli et al., 1994).



Figure 1.6: Biosynthetic pathway of the activated sialic acid in pathogenic bacteria.

1.3.1 UDP-GlcNAc 2-epimerase

The first committed step of sialic acid biosynthesis in pathogenic bacteria is catalyzed by hydrolyzing UDP-GlcNAc 2-epimerase, which converts UDP-GlcNAc into ManNAc (Murkin *et al.*, 2004, Vann *et al.*, 2004). Recently, a mechanism for UDP-GlcNAc 2epimerase from *Neisseria meningitidis* was proposed involving an *anti* elimination of UDP to form 2-acetamidoglucal as a reaction intermediate, followed by the *syn* addition of water to generate the final product ManNAc (Murkin *et al.*, 2004) (Figure 1.7). The overall stereochemistry involves the inversion of configuration at C-2 and the retention of configuration at C-1 upon C–O bond cleavage. Proposed mechanism suggests that the unidentified catalytic base of the enzyme attacks at H-2, followed by the release of UDP.
This initial step generates the reaction intermediate, 2-acetamidoglucal, which is subsequently hydrolyzed by water to form ManNAc. Site-directed mutagenesis of three potential active site residues (D100N, E122Q, and D131N) revealed that mutations on these residues dramatically reduce activities, implying their roles in catalysis. Additional NMR spectroscopic analysis confirmed that both E122Q and D131N mutants were able to generate 2-acetamidoglucal as the first reaction product, however, the production of ManNAc was observed only after significantly extended time frame (7 days) (Murkin *et al.*, 2004). In addition, 2-acetamidoglucal was not detected in the reaction with D100N mutant, which highlights a possibility that Asp-100 is the potential candidate as a catalytic base of this enzyme.



Figure 1.7: Proposed mechanism of bacterial hydrolyzing UDP-GlcNAc 2-epimerase.

1.3.2 Sialic Acid Synthase

Sialic acid synthase catalyzes the condensation of phosphoenolpyruvate (PEP) with ManNAc to generate NeuNAc (sialic acid) in pathogenic bacteria (Masson & Holbein, 1983, Annunziato *et al.*, 1995, Vann *et al.*, 1997). The proposed mechanism of sialic acid synthase involves the attack by C-3 of PEP onto the carbonyl group of the open form of ManNAc, which is facilitated by the metal ion that serves as an electrophilic catalyst to activate the carbonyl group of ManNAc (Gunawan *et al.*, 2005). This initial step generates an oxocarbenium ion which is readily reacted with water to form a tetrahedral reaction intermediate, followed by the loss of phosphate group to produce the open chain form of sialic acid that cyclizes spontaneously in solution (Figure 1.8).



Figure 1.8: Proposed mechanism of sialic acid synthase.

The crystal structure of the sialic acid synthase from *Neisseria meningitidis* in complex with *N*-acetylmannositol (reduced ManNAc), PEP, and Mn²⁺ revealed that this enzyme has a unique domain-swapped homodimeric features (Gunawan *et al.*, 2005). Each monomer of the enzyme can be divided into two domains that are joined by an extended linker region. The C-terminal domain has a typical TIM-barrel ((α/β)₈ barrel) fold with an eight-stranded β -barrel enclosed by eight α -helices. The N-terminal region forms an antifreeze-like domain, which shares high degree of sequence identity (~40%) with the type III antifreeze protein and contributes to the domain-swapped homodimeric quaternary structure (Figure 1.9) (Baardsnes & Davies, 2001).



Figure 1.9: Overall structure of sialic acid synthase.

(A) Ribbon diagram of the monomer of sialic acid synthase showing TIM-barrel and antifreeze-like domains. Helix, sheet, and loop are shown in red, yellow, and green, respectively. (B) Domain-swapped homodimeric structure of sialic acid synthase with each monomer colored in red and blue, respectively. Bound substrates are shown as stick models with cyan for carbon atoms, and non-carbon atoms are colored according to atom type (nitrogen, blue; oxygen, red; and phosphorus, orange).

1.3.3 CMP-NeuNAc Synthetase

CMP-NeuNAc (CMP-*N*-acetylneuraminic acid) synthetase is the enzyme that transfers cytidine-5'-monophosphate (CMP) to the synthesized sialic acid, thereby generating an activated sialic acid molecule (CMP-NeuNAc) which is the universal donor substrate for all known sialyltransferases (Figure 1.10) (Warren & Blacklow, 1962, Vann *et al.*, 1987). Furthermore, it is an essential enzyme for the expression of the capsular polysialic acids at the surface of pathogenic bacteria, which is confirmed by gene knockout studies (Haft *et al.*, 1996). CMP-NeuNAc synthetase catalyzes the direct nucleophilic attack of the anomeric oxygen at C-2 position of the sialic acid on the α -phosphate of cytidine-5'-triphosphate (CTP) to produce the activated sialic acid in the presence of a divalent cation (Ambrose *et al.*, 1992, Mizanur & Pohl, 2008).



Figure 1.10: Reaction of CMP-NeuNAc synthetase.

The crystal structure of CMP-NeuNAc synthetase from *Neisseria meningitidis* in complex with substrate analogue, CDP (cytidine diphosphate), revealed the homodimeric nature of this enzyme. Each monomer is composed of a major globular domain, which is structurally classified as $\alpha\beta\alpha$ sandwich fold and a small minor domain participated in the dimerization (Figure 1.11A, B). Structural analysis of the nucleotide binding site revealed that this enzyme undergoes significant conformational rearrangements in the active site upon substrate binding. Structural motifs including the P-loop (residues 10–22) and the neighboring helix (residues 71–80) disordered in the apo enzyme structure adopt to well ordered conformations upon the binding of the substrate analogue (CDP) (Figure 1.11C, D) (Mosimann *et al.*, 2001).

1.4 Transfer, Polymerization, and Modification of Sialic Acid in Pathogenic Bacteria

Activated sialic acid (CMP-NeuNAc) is subsequently transferred by sialyltransferases to form lipooligosaccharides (LOS) or capsular polysaccharides (CPS) in pathogenic bacteria, providing a major virulence determinant on pathogenesis (Steenbergen & Vimr, 1990, Smith *et al.*, 1995, Gilbert *et al.*, 2000). Furthermore, polymerized sialic acids can be modified by *O*-acetyltransferases in several pathogenic bacteria, which make those bacterial pathogens more virulent than non-*O*-acetylated strains (Orskov *et al.*, 1979, Frasa *et al.*, 1993, Lemercinier & Jones, 1996).



Figure 1.11: Overall structure of CMP-NeuNAc synthetase.

(A) Ribbon diagram of the monomer of CMP-NeuNAc synthetase showing a major domain composed of $\alpha\beta\alpha$ sandwich fold and a small minor domain participated in the dimerization. Helix, sheet, and loop are shown in blue, yellow, and magenta, respectively. (B) Homodimeric quaternary structure of CMP-NeuNAc synthetase with each monomer colored in red and green, respectively. Bound substrate analogues (CDP) are shown as stick models with gray for carbon atoms, and non-carbon atoms are colored according to atom type (nitrogen, blue; oxygen, red; and phosphorus, orange). (C) Active site of apo CMP-NeuNAc synthetase in the absence of CDP. Helix, sheet, and loop are shown in cyan, yellow, and gray, respectively. (D) Active site of CMP-NeuNAc synthetase in complex with CDP. Helix, sheet, and loop are shown in blue, yellow, and gray, respectively. The P-loop and the neighboring helix are shown in red and green, respectively.

1.4.1 Sialyltransferase

The of sialylated glycans bacterial surfaces biosynthesis on such as lipooligosaccharides is catalyzed by sialyltransferases, which transfer sialic acids to the growing chain of oligosaccharides on the surface of pathogenic bacteria (Smith et al., 1995, Kahler & Stephens, 1998). They utilize an activated sialic acid (CMP-NeuNAc) as the universal donor substrate to sialylate various acceptor substrates including galactose, Nacetylgalactosamine, or another sialic acid with distinct linkages based on their specificities (Harduin-Lepers et al., 1995, Angata & Varki, 2002). Sialyltransferases are classified into several families of glycosyltransferases (GT) in the CAZy database (Carbohydrate-Active enZyme, http://www.cazy.org) based on their amino acid sequence similarities (Coutinho et al., 2003, Cantarel et al., 2009). Currently, bacterial sialyltransferases have been classified into four distinct families including GT38, GT42, GT52, and GT80 (Table 1.3). They are all inverting glycosyltransferases, which catalyze the transfer reactions with the inversion of stereochemistry at the anomeric center by S_N 2-like direct displacement mechanism via a single oxocarbenium ion-like transition state (Figure 1.12) (Lairson et al., 2008). An active site residue serves as catalytic base to deprotonate the incoming nucleophile of the acceptor substrate, facilitating the catalytic reaction.

CAZy	Species	Known Activities	Statistics
GT38	Neisseria meningitidis $(14)^*$	α-2,8/α-2,9-	CAZy entries: 19
	Escherichia coli (5)	polysialyltransferase	GenBank [•] : 25
			UniProt [‡] : 19
GT42	Campylobacter jejuni (55)	α -2,3/ α -2,8-	CAZy entries: 86
	Haemophilus influenzae (10)	sialyltransferase	GenBank: 150
	Helicobacter acinonychis (2)		UniProt: 64
	Pasteurella multocida (1)		3D: 2 (PDB [¶] : 5)
GT52	Aeromonas punctata (1)	α -2,3-sialyltransferase	CAZy entries: 61
	Citrobacter koseri (1)		GenBank: 69
	Edwardsiella ictaluri (1)		UniProt: 55
	Haemophilus influenzae (5)		3D: 1 (PDB: 6)
	Haemophilus somnus (1)		
	Neisseria gonorrhoeae (16)		
	Neisseria meningitidis (6)		
	Pasteurella multocida (1)		
	<i>Psychrobacter arcticus</i> (1)		
	Shigella boydii (1)		
	Streptococcus agalactiae (1)		
GT80	Haemophilus ducreyi (1)	α -2,3/ α -2,6-	CAZy entries: 10
	Pasteurella multocida (2)	sialyltransferase	GenBank: 12
	Photobacterium damselae (1)		UniProt: 6
	Photobacterium leiognathi (1)		3D: 4 (PDB: 16)
	Photobacterium phosphoreum (1)		
	Photobacterium sp. (2)		
	Shewanella piezotolerans (1)		
	Vibrio sp. (1)		

Table 1.3: Bacterial sialyltransferase families in the CAZy database.

* The number inside the bracket corresponds to the specific number of sequences in the CAZy database identified for that species.

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/
UniProt, http://www.uniprot.org/
Protein Data Bank, http://www.pdb.org/



Figure 1.12: Proposed mechanism of the sialyltransferase.

The sialyltransferase untilizes a S_N 2-like direct displacement mechanism, resulting the inversion of configuration at the anomeric center via a single oxocarbenium ion-like transition state.

The crystal structure of sialyltransferase Cst-II (GT42) isolated from the human pathogen *Campylobacter jejuni* is the first reported structure of sialyltransferase (Chiu *et al.*, 2004). It is a bifunctional enzyme, which transfers the first sialic acid residue to the terminal galactose moiety of the lipooligosaccharide with α -2,3-linkage and subsequently transfers the second sialic acid to the initially formed sialylated lipooligosaccharide with α -2,8-linkage (Figure 1.13). Structural analysis of Cst-II revealed that it has a modified GT-A fold, which is composed of C-terminal flexible lid domain and N-terminal single Rossmann nucleotide binding domain without the conserved DXD motif (Figure 1.14). Additional kinetic characterizations of Cst-II have identified several key residues involved in the substrate binding and catalysis including the potential catalytic base, His-188 (Chiu *et al.*, 2004).



Figure 1.13: Reaction scheme of the sialyltransferases from C. jejuni.

Sialyltransferases from *Campylobacter jejuni* utilize CMP-NeuNAc as a universal donor substrate and galactose derivatives as acceptor substrates. The bonds generated by monofunctional (Cst-I, Cst-II_{OH19}) and bifunctional (Cst-II_{OH4384}) enzymes are highlighted by dotted green circles. Monofunctional Cst-II_{OH19} and Cst-I can only catalyze the first transfer reaction onto the galactose residue while bifunctional Cst-II_{OH4384} can also catalyze the second transfer onto the synthesized sialoside from the first transfer reaction.



Figure 1.14: Overall structure of the sialyltransferase Cst-II.

The structure of Cst-II monomer is shown with the N-terminal Rossmann domain and the lid domain. The flexible lid domain is colored in red, and the potential catalytic base (His-188) is shown as a stick model. Carbon atoms are shown in yellow and green for donor analogue (CMP-3FNeuNAc) and the catalytic base, respectively. Non-carbon atoms are colored according to atom type (nitrogen, blue; oxygen, red; phosphorus, orange; and fluorine, gray).

Cst-I from human pathogen *Campylobacter jejuni* is another well-characterized sialyltransferase in GT42 family. Cst-I is a monofunctional enzyme, which transfers a single sialic acid residue to the terminal galactose moiety of the lipooligosaccharide of the bacterial pathogen with α -2,3-linkage. Structural characterizations of Cst-I showed that it has an identical fold observed in the structure of the bifunctional Cst-II including an N-terminal Rossmann nucleotide binding domain, and a C-terminal flexible lid domain which encompasses the active site of the enzyme (Figure 1.15) (Chiu *et al.*, 2007). Sequence

alignment of bifunctional Cst-II and monofunctional Cst-I shows ~40% overall sequence identity with a number of conserved residues involved in the donor binding and catalysis.



Figure 1.15: Overall structure of Cst-I and its structural alignment with Cst-II. (A) Overall structure of Cst-I in complex with its donor substrate analogue (CMP-3FNeuNAc). The lid domain is colored in cyan and the donor analogue is shown as a stick model. (B) Structural alignment between Cst-I and Cst-II. Lid domains of Cst-I and Cst-II are colored in cyan and red, respectively. Carbon atoms of donor analogue for Cst-I and Cst-II are colored in yellow and magenta, respectively. Non-carbon atoms are colored according to atom type (nitrogen, blue; oxygen, red; phosphorus, orange; and fluorine, gray).

Recently reported structure of *Pm*ST1 isolated from *Pasteurella multocida* is the first example of sialyltransferase with a typical GT-B fold, composed of two separate Rossmann domains with the substrate binding site in the deep cleft between two domains (Figure 1.16) (Ni *et al.*, 2007). *Pm*ST1 is a multifunctional enzyme, which is able to catalyze both α -2,3 and α -2,6 sialyltransfer reactions as well as α -2,3-sialidase, and α -2,3-*trans*-sialidase reactions. The major function of the *Pm*ST1 is α -2,3-sialyltransferease, transferring the sialic acid residue from activated sugar donor (CMP-NeuNAc) to the terminal galactose moiety of the acceptor (Yu *et al.*, 2005). Structural and kinetic analysis of PmST1 revealed both donor and acceptor binding characteristics, and identified essential residues for enzyme activity including the proposed catalytic base, Asp-141 (Ni *et al.*, 2007).



Figure 1.16: Overall structure of *Pm*ST1.

Overall structure of *Pm*ST1 in complex with donor sugar analogue (CMP-3FNeuNAc) and acceptor analogue (lactose) is represented with discrete color schemes for secondary structural elements. For the first Rossmann domain, helix and sheet are shown in red and yellow, respectively. For the second Rossmann domain, helix and sheet are shown in cyan and magenta, respectively. Loops are shown in gray. Bound substrates are shown as stick models using yellow and green for carbon atoms of donor and acceptor analogues, respectively. Non-carbon atoms are colored according to atom type (nitrogen, blue; oxygen, red; phosphorus, orange; and fluorine, gray).

1.4.2 Polysialyltransferase

Polysialyltransferases catalyze the processive polysaccharide chain elongation in pathogenic bacteria, successively transferring sialic acid residues from the activated donor (CMP-NeuNAc) to the nonreducing terminus of the growing chain of polysialic acids, and are the major determinant of serotype specificities in various bacterial pathogens (Table 1.4) (Steenbergen & Vimr, 1990, McGowen et al., 2001, Steenbergen & Vimr, 2003, Willis et al., 2008). Recent biochemical characterizations for the bacterial polysialyltransferase from *Neisseria meningitidis* serogroup B revealed that the C-terminal domain is indispensable for enzymatic activity, and also identified two functional motifs (D/E-D/E-G and HP motifs) conserved in various bacterial sialyltransferases (Freiberger et al., 2007). Subsequent sitedirected mutagenesis and kinetic analysis showed that residues in these motifs are critical for enzymatic activity and revealed the importance of the HP motif in donor binding (Freiberger et al., 2007). Neisseria meningitidis serogroup W-135 and Y express heteropolymeric sialic acids composed of α -2,6-linked N-acetylneuraminic acid with galactose and glucose, respectively (Table 1.4) (Bhattacharjee et al., 1976). Corresponding genes encoding polysialyltransferases in those meningococcal serogroups were identified and biochemically characterized showing both hexosyltransferase and sialyltransferase activities (Claus et al., 1997, Claus et al., 2009). It was also found that their acceptor specificities for hexosyltransfer reactions were controlled by a single amino acid at position 310, whereas proline residue determines galactosyltransferase activity and glycine residue determines glucosyltransferase activity, resulting W-135 and Y capsular polysialic acids, respectively (Claus et al., 2009).

Bacterial species	Serotype specificities
E. coli K1	$[\alpha-2,8-\text{Neu5Ac}]_n$
E. coli K92	$[\alpha - 2, 8/2, 9 - \text{Neu5Ac}]_n$
N. meningitidis B	$[\alpha-2,8-\text{Neu5Ac}]_n$
N. meningitidis C	$[\alpha-2,9-\text{Neu5Ac}]_n$
N. meningitidis W-135	[6-Galα-1,4-Neu5Ac-2] _n
N. meningitidis Y	$[6-Glc\alpha-1,4-Neu5Ac-2]_n$

Table 1.4: Serotype specificities of pathogenic bacteria.

1.4.3 Polysialic Acid O-acetyltransferase

Synthesized and polymerized sialic acids can be modified by *O*-acetylations in several pathogenic bacteria including *E. coli* K1 and certain serogroups of *N. meningitidis*, which alters the physiochemical properties of the polysaccharides of those pathogens (Bhattacharjee *et al.*, 1976, Jennings *et al.*, 1977, Orskov *et al.*, 1979, Lemercinier & Jones, 1996). This modification process is specifically catalyzed by polysialic acid *O*-acetyltransferases in various bacterial pathogens (Higa & Varki, 1988, Bergfeld *et al.*, 2007, Bergfeld *et al.*, 2009). Genetic characterizations of polysialic acid *O*-acetyltransferases reported to date includes *neuO* in *E. coli* K1, *oatC* and *oatWY* in *N. meningitidis*, which are indispensable for *O*-acetylations of polysialic acids in those pathogenic bacteria (Claus *et al.*, 2004, Deszo *et al.*, 2005). Polysialic acids expressed in *E. coli* K1 are acetylated at O-7 and O-9 positions of sialic acids (Deszo *et al.*, 2005). In addition, the polysialic acid is also acetylated at the O-7 or O-8 hydroxyl group in *N. meningitidis* serogroup C, whereas the O-7 or O-9 position is acetylated in serogroup W-135 and Y of *N. meningitidis* (Lemercinier & Jones, 1996). Recent biochemical analysis of NeuO from *E. coli* K1

predicted that this enzyme belongs to the left-handed β -helical fold, and identified two essential catalytic residues (His-119, Trp-143) (Bergfeld *et al.*, 2007). Interestingly, OatC enzyme from *N. meningitidis* serogroup C was predicted that it belongs to the α/β -hydrolase family with the catalytic triad composed of Ser-286, Asp-376, and His-399 (Bergfeld *et al.*, 2009).

1.5 Transport of the Polysialic Acids in Pathogenic Bacteria

Synthesized and polymerized sialic acids are transported from their cytoplasmic sites of synthesis through inner/outer membranes of the pathogenic bacteria by a variety of proteins involved in the capsular polysaccharide transport (Figure 1.17) (Bliss & Silver, 1996, Whitfield, 2006, Vimr & Steenbergen, 2009). One of the most extensively studied organisms for the capsular polysaccharide transportation is *Escherichia coli* K1, which contains 17 kb capsular polysaccharide gene cluster divided by three functional regions (region 1, 2, and 3) (Figure 1.18A) (Vimr *et al.*, 1989). The flanking regions 1 and 3 encode the strongly conserved genes whose products are responsible for the polysaccharide transport in the bacterial pathogen (Roberts, 1995, Vimr *et al.*, 1995). These regions have essential roles in capsular polysaccharide transport procedures as cells harboring mutations in these regions resulted in the intracellular accumulation of unexported polysaccharides (Vimr *et al.*, 1995, Vimr & Steenbergen, 2009).



Figure 1.17: Model of the capsular polysaccharide biosynthesis and transport.

Gene product indicated by colored protein modules are defined in Figure 1.17 and Table 1.5 (SiaA: UDP-GlcNAc 2-epimerase, SiaB: CMP-Neu5Ac synthase, SiaC: sialic acid synthase, SiaD: polysialyltransferase, OatWY: polysialic acid *O*-acetyltransferase, CtrA: outer membrane protein, CtrB: periplasmic connector, CtrC: inner membrane transporter, CtrD: ATP-binding component). Gray and orange small circles indicate unacetylated and acetylated sialic acid, respectively.

Furthermore, homologous proteins have been identified in other pathogenic bacteria including *Neisseria meningitidis*, *Haemophilus influenzae*, and *Salmonella typhi*, and all of which are suggested to play similar roles in the polysaccharide transport (Table 1.5) (Figure 1.18B) (Kroll *et al.*, 1990, Frosch *et al.*, 1991, Hashimoto *et al.*, 1993, Bliss & Silver, 1996).

A. Genetic organization of	the capsular polysialic acid gene c	luster in <i>E. coli</i> K1
├─── Region 3───┤	Region 2	Region 1 ———
kpsM kpsT neuD neu	B neuA neuC neuE neuS	kpsS kpsC kpsU kpsD kpsE kpsF
Proposed Functions		
KpsM - ABC-Transporter KpsT - ATPase NeuD - Acetyltransferase NeuB - NeuNAc synthese NeuA - CMP-NeuNAc synthethase NeuC - UDP-GICNAc 2-epimerase NeuE - Unknown NeuS - Polysialyltransferase B. Genetic organization of Region C	KpsS - Unknown KpsC - Unknown KpsD - CMP-KDO synthetase KpsD - Polysaccharide transport KpsF - Polysaccharide transport KpsF - Arabinose 5-phosphate isomerase the capsular polysialic acid gene clipping Region A Image: Region A SiaA SiaB SiaA SiaC	uster in <i>N. meningitidis</i> B
Proposed Functions		
CtrD - ABC-Transporter CtrC - ABC-Transporter CtrB - Polysaccharide transport CtrA - Polysaccharide transport LipB - Unknown LipA - Unknown	SiaA - UDP-GIcNAc 2-epimerase SiaB - CMP-NeuNAc synthetase SiaC - NeuNAc synthase SiaD - Polysialyltransferase	

Figure 1.18: Gene clusters of the capsular polysaccharide biosynthesis and transport in *E. coli* K1 and *N. meningitidis* B.

<i>E. coli</i> K1	N. meningitidis	H. influenza	S. typhi
KpsM	CtrC	VexB	BexB
KpsT	CtrD	VexC	BexA
KpsE	CtrB	VexD	BexC
KpsD	N/A^a	N/A^a	N/A^{a}
N/A^b	CtrA	VexA	BexD

Table 1.5: Homologues of capsular polysaccharide transport proteins in pathogenic bacteria.

^{*a*} The capsular gene clusters do not encode *kps*D homologues in those pathogens.

^b The capsular gene cluster does not encode *ctr*A homologue in *E. coli* K1.

1.5.1 Proteins involved in the Transport of Polysialic Acids across the Inner Membrane

The region 3 of the capsular polysaccharide gene cluster in *E. coli* K1 encodes two distinctive genes, *kps*M and *kps*T, whose products constitute a ATP-binding cassette (ABC) transporter involved in the transport of polysaccharides through the cytoplasmic membrane (Pavelka *et al.*, 1991, Bliss & Silver, 1996). Mutational analysis revealed that cells lacking either *kps*M or *kps*T are unable to transport the synthesized polysaccharides to the cell surface and accumulate intracellular polysaccharides at the periphery of the cytoplasm close to the inner membrane, indicating these proteins are responsible for the transportation of polymers across the inner membrane (Pavelka *et al.*, 1991, Pavelka *et al.*, 1994, Bliss *et al.*, 1996). KpsM and KpsT are proposed to function as homodimers, which transport synthesized polysaccharides across the cytoplasmic membrane utilizing energy from ATP hydrolysis with conformational changes (Bliss & Silver, 1996, Nsahlai & Silver, 2003). Homologues of KpsMT have been described in other encapsulated bacteria such as CtrCD of *N. meningitidis*, and proposed to have similar roles in the polysaccharide transport (Frosch *et al.*, 1991).

1.5.1.1 Inner Membrane Transporter KpsM

KpsM is a hydrophobic, integral inner membrane protein composed of six predicted trans-membrane spanning regions with its N- and C-termini localized to the cytoplasm. Topological and mutational analyses have revealed that the third periplasmic loop and the first cytoplasmic loop are essential for the function of KpsM (Figure 1.19) (Pigeon & Silver, 1994). The first cytoplasmic loop contains the conserved EAA motif, which is proposed to be involved in the interaction with the ATP-binding component (KpsT) during the polymer transport, inducing conformational changes.



Figure 1.19: Topological model of KpsM in the inner membrane.

Predicted trans-membrane domains are shown as green bars. The periplasmic loops (PI, PII, and PIII) are shown in black, and the cytoplasmic loops (CI, CII) are shown in blue.

1.5.1.2 ATP-Binding Component KpsT

KpsT is a hydrophilic, peripheral inner membrane protein containing the ATP-binding site. It is the essential component of the polysaccharide transport machinery in encapsulated pathogenic bacteria (Pavelka *et al.*, 1991, Pavelka *et al.*, 1994, Bliss *et al.*, 1996). KpsT binds to ATP, undergoes an ATP-dependent conformational change, and hydrolyzes ATP for the polymer translocation across the inner membrane (Pavelka *et al.*, 1994, Nsahlai & Silver, 2003). Further biochemical analysis has shown that KpsM tends to be more susceptible to the proteolytic degradation in the presence of KpsT, indicating the conformational change upon the binding of KpsT (Nsahlai & Silver, 2003).

1.5.2 Proteins involved in the Transport of Polysialic Acids across the Outer Membrane

Region 1 of the capsular polysaccharide gene cluster of *E. coli* K1 encodes a number of genes associated with the transport of synthesized polymers (Figure 1.18A) (Vimr *et al.*, 1989). Among these genes, *kps*D and *kps*E have roles in the transportation of polysaccharides across the outer membrane of the bacteria. Mutational analyses have shown that mutations in *kps*D or *kps*E result in the accumulation of unexported polysaccharides in the periplasm, indicating these genes are involved in the polymer transport through the outer membrane (Silver et al., 1987, Bronner et al., 1993, Pazzani et al., 1993). Homologous genes of *kps*E have been described in other encapsulated bacteria such as CtrB of *N. meningitidis* and proposed to have a similar role in the polysaccharide translocation. Interestingly, *kps*D has no known homologue, suggesting a unique

polysaccharide transport system of *E. coli* (Bliss & Silver, 1996). Similarly, the gene that encodes the outer membrane component CtrA in *N. meningitidis* has been described. It has no known homologue to any *E. coli* protein associated with the polysaccharide transport, suggesting that polymer transport machinery across the outer membrane has been evolved differently in two distinct bacterial pathogens (Frosch *et al.*, 1992).

1.5.2.1 Outer Membrane Protein KpsD

KpsD is an essential outer membrane protein involved in the transport of polysaccharides across the outer membrane in encapsulated E. coli (McNulty et al., 2006). Mutational analysis has revealed that cells harboring the mutation in kpsD accumulate the unexported polysaccharide in the periplasm, indicating the product of kpsD is intimately involved in the polymer translocation through the outer membrane of the encapsulated bacteria (Silver et al., 1987). Subsequent biochemical analysis have shown that KpsD is detectable in outer membrane fractions and its membrane association is dependent on KpsE, suggesting the translocation through the periplasm is operated by the orchestrating action of KpsD and KpsE in conjunction with KpsMT transporter (Arrecubieta et al., 2001, McNulty et al., 2006). Hydropathy plotting with the fluorescence-activated cell sorting (FACS) analysis using KpsD antiserum proposed a possible topology for KpsD in which the Nterminal domain is embedded in the outer membrane with a large C-terminal domain in the periplasm (McNulty *et al.*, 2006). Further biochemical analysis revealed that KpsD has a dimeric feature in contrast to other outer membrane proteins, which form higher oligomers in the outer membrane (Nesper et al., 2003, Beis et al., 2004, McNulty et al., 2006).

1.5.2.2 Periplasmic Connector KpsE

KpsE is a membrane fusion protein linking the KpsMT transporter to the periplasmic domain of the outer membrane protein KpsD to facilitate the translocation of the polysaccharide in *E. coli* (Bliss & Silver, 1996, Arrecubieta *et al.*, 2001, Vimr & Steenbergen, 2009). Cells harboring the mutation in *kps*E have been shown to accumulate the polymer in the periplasm, indicating the importance of KpsE in the polysaccharide transport through the periplasm (Bronner *et al.*, 1993). Biochemical analyses on KpsE have revealed the location of the protein in the periplasm and the association with the inner membrane via a C-terminal amphipathic α -helix in which hydrophobic residues of the helix are buried in the interior of the inner membrane and the hydrophilic residues are exposed to the periplasmic space (Rosenow *et al.*, 1995, Arrecubieta *et al.*, 2001, Phoenix *et al.*, 2001).

1.5.2.3 Outer Membrane Protein CtrA

CtrA is the dedicated outer membrane protein involved in the transport of the capsular polysaccharide in *N. meningitidis*. This essential outer membrane protein is exclusively expressed in *N. meningitidis*, and is highly conserved with nearly identical amino acid sequences (98% identity) in all meningococcal serotypes independent of chemical compositions of capsular polysaccharides found in various serogroups including A, B, C, W-135, and Y (Frosch *et al.*, 1992). CtrA is proposed to have a porin-like β -barrel structure composed of eight-membrane spanning β -strands anchored in the outer membrane of the bacteria. Epitope mapping with a monoclonal antibody have revealed that CtrA is predicted to have a C-terminal periplasmic domain composed of amino acids 197–259

(Frosch et al., 1992).

1.6 Objectives of Thesis

Sialic acid plays a vital role in various biological processes including cellular recognition and adhesion. The metabolism of sialic acid is particularly important to host-pathogen interactions as many virulent bacteria utilize sialic acids in order to evade the host's immune system. The importance of sialic acids in pathogenic bacteria as means of evading the host's immune response, makes essential proteins involved in the sialic acid metabolism an attractive target for the development of novel antimicrobial compounds. The objective of this thesis is to understand the structure and kinetic mechanism of a series of essential enzymes involved in the sialic acid metabolism in bacterial pathogens.

Chapter 2 describes the synthesis and evaluation of the first potent inhibitor of bacterial sialic acid synthase NeuB from *N. meningitidis*. Crystallographic and kinetic analyses on sialic acid synthase in complex with the inhibitor are presented. Detailed binding characteristics of the inhibitor are analyzed to examine the inhibitory mechanism, which provide important insights into the mechanism of the sialic acid synthase in pathogenic bacteria. This work has been published in *Biochemistry* (Liu *et al.*, 2009).

Chapter 3 describes the structural and kinetic characterization of polysialic acid *O*acetyltransferase OatWY from *N. meningitidis* in apo form and in complex with either donor substrate (acetyl-CoA) or analogues (CoA, *S*-(2-oxopropyl)-CoA). Crystallographic, kinetic, and mutagenesis studies are presented to reveal substrate specificities and essential residues for the catalytic mechanism of this enzyme. This work has been published in

Journal of Biological Chemistry (Lee et al., 2009).

Chapter 4 describes the structural and kinetic characterization of sialyltransferase Cst-II isolated from *C. jejuni* in complex with the fragment of the donor substrate, CMP and the terminal trisaccharide of its natural acceptor, Neu5Ac- α -2,3-Gal- β -1,3-GalNAc. Crystallographic and kinetic analyses of the wild type enzyme and active site mutants are presented. This work has generated a manuscript and will be submitted for publication.

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<u>CHAPTER 2</u>: INHIBITION OF *NEISSERIA MENINGITIDIS* SIALIC ACID SYNTHASE BY A TETRAHEDRAL INTERMEDIATE ANALOGUE *

2.1 Introduction

Sialic acid or N-acetylneuraminic acid (NeuAc) is a nine carbon α -keto acid that plays a wide variety of important biological roles (Angata & Varki, 2002, Schauer, 2004). In mammals, it is found as the terminal carbohydrate residue of many cell surface glycoconjugates. Thus, its structure defines the periphery of mammalian cells, and it is a key determinant in mediating cellular recognition processes (Varki, 1997, Traving & Schauer, 1998). These include the binding between selectins and leukocytes in the inflammation response and the binding of the influenza virus and mammalian cells during infection (Olofsson & Bergstrom, 2005, Varki, 2007). While sialic acid is not commonly found in prokaryotes, certain pathogenic bacteria biosynthesize it as a virulence factor (Severi et al., 2007). Infections by Neisseria meningitidis and Escherichia coli K1 are among the leading causes of bacterial meningitis (Saez-Llorens & McCracken, 2003). These bacteria produce a capsular polysaccharide comprised of polysialic acid as a means of evading the host's immune system (Finne, 1985). Since polysialic acid is present as a posttranslational modification of neural cell adhesion molecules (NCAM's), the capsular polysaccharide allows the bacterial cells to appear "human-like" via an act of molecular mimicry (Kleene & Schachner, 2004). Sialic acid is also found as a component of the

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lipooligosaccharide in Campylobacter jejuni (Preston et al., 1996).

In bacteria, sialic acid is synthesized in two steps from UDP-*N*-acetylglucosamine (UDP-GlcNAc) (Tanner, 2005). The first step is catalyzed by a hydrolyzing UDP-GlcNAc 2-epimerase that generates *N*-acetylmannosamine (ManNAc) and UDP (Murkin *et al.*, 2004, Vann *et al.*, 2004). Sialic acid synthase then condenses ManNAc and phosphoenolpyruvate (PEP) to generate *N*-acetylneuraminic acid (sialic acid, Figure 2.1) (Blacklow & Warren, 1962, Vann et al., 1997, Hwang et al., 2002, Suryanti et al., 2003, Sundaram et al., 2004, Gunawan et al., 2005).



Figure 2.1: Proposed mechanism of the reaction catalyzed by sialic acid synthase (NeuB or *N*-acetylneuraminic acid synthase). The insert shows the structure of inhibitor 1.

In mammals, the biosynthesis differs in that ManNAc is first phosphorylated to give ManNAc 6-phosphate, and then the corresponding synthase generates *N*-acetylneuraminic acid 9-phosphate (Hinderlich *et al.*, 1997, Stasche *et al.*, 1997, Lawrence *et al.*, 2000, Chen *et al.*, 2002, Chou *et al.*, 2003, Hao *et al.*, 2006). Mechanistic studies on the bacterial sialic acid synthase have shown that it requires a divalent metal ion for catalysis (Blacklow & Warren, 1962, Sundaram et al., 2004, Gunawan et al., 2005). The enzyme is thought to catalyze the ring opening of ManNAc to expose the aldehyde functionality at C-1 (Figure 2.1). The C-3 carbon of PEP then attacks the aldehyde to form an oxocarbenium ion intermediate. This is facilitated by the metal ion that serves as an electrophilic catalyst and activates the carbonyl of the aldehyde toward attack. Water then adds to the oxocarbenium ion to generate a tetrahedral intermediate. A subsequent loss of phosphate gives the open chain form of sialic acid that will readily cyclize to the pyranose form in solution.

Support for this mechanism comes from studies on the *N. meningitidis* sialic acid synthase, NeuB (Gunawan et al., 2005). When PEP bearing an ¹⁸O-isotopic label in the phosphate bridging position ([2-¹⁸O]-PEP) was used as a substrate, the enzymatic reaction generated ¹⁸O-labeled phosphate as a product. This confirms that the loss of phosphate proceeds via a C–O bond cleavage mechanism, as is proposed to occur during the collapse of the tetrahedral intermediate. A crystallographic analysis of the synthase in a complex with *N*-acetylmannositol (substrate reduced at C-1), PEP, and Mn²⁺ also supports the mechanism (Gunawan et al., 2005). The use of the reduced substrate prevents the reaction from occurring and provides an excellent model for the Michaelis complex formed during

catalysis. The C-1 hydroxyl of the substrate analogue is coordinated to Mn^{2+} , as would be expected if the normal role of the metal ion were to activate the C-1 aldehyde via electrostatic catalysis. The *si* face of the PEP is positioned directly above the *pro-S* hydrogen of *N*-acetylmannositol in the observed structure. This is consistent with a stereochemical analysis of the reaction catalyzed by the *C. jejuni* enzyme (Sundaram et al., 2004). Deuterium-labeled PEP was employed to show that the *si* face of PEP adds to the *si* face of the ManNAc aldehyde.

This work describes the synthesis and evaluation of an inhibitor that mimics the tetrahedral intermediate formed in the sialic acid synthase reaction (compound **1**, Figure 2.1 insert). Removal of the C-2 hydroxyl imparts stability to the compound without increasing steric bulk or altering the distribution of charges within the molecule. The compound was generated as a mixture of stereoisomers at C-2 in order to investigate the preferred orientation of groups within the active site of the enzyme. Crystallographic analysis of a complex between sialic acid synthase and the more tightly binding stereoisomer of compound **1** shows that the inhibitor bears an (*R*)-configuration at C-2. This suggests that the tetrahedral intermediate bears an (*R*)-configuration at C-2 and that a metal-bound hydroxide is delivered to the *si* face of the oxocarbenium ion intermediate during catalysis.

2.2 Experimental Procedures

2.2.1 Materials and General Methods

Chemicals were purchased from Sigma-Aldrich and used without further purification unless otherwise noted. 2-Amino-6-mercapto-7-methylpurine ribonucleoside was purchased from Berry and Associates. Bacterial purine nucleoside phosphorylase was purchased from Sigma (one unit will cause the phosphorolysis of 1.0 µmol of inosine to hypoxanthine and ribose 1-phosphate per minute at pH 7.4, 25 °C) (Bradford, 1976). Pyridine and triethylamine were distilled over CaH₂ under an atmosphere of N₂. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard. ¹H and ³¹P NMR spectra were obtained on Bruker AV400 NMR spectrometers. Mass spectra were obtained on a Waters Micromass LCT mass spectrometer using electrospray ionization (ESI-MS).

2.2.2 Synthesis of Inhibitor 1

Methyl 5-Acetamido-3,5-dideoxy-2-methylidine-4,6,7,8,9-penta-*O*-acetyl-D-*glycero*-D*galacto*-2-nonulosonate (3)

Compound **2** was prepared in three steps from ManNAc as described previously (Figure 2.2) (Weitz & Bednarski, 1989). To a solution of compound **2** (3.72 g, 8.9 mmol) in ACN/HCl (60 mL, 20:1) were added methyl bromomethylacrylate (0.6 mL, 5 mmol) and a suspension of indium powder (100–200 mesh, 1.72 g, 15 mmol) in ACN/HCl (5 mL, 20:1). The mixture was vigorously stirred at 45 °C for 3.5 h, and then the indium clump was removed. Fresh methyl bromomethylacrylate (0.6 mL, 5 mmol) and a suspension of

indium (0.5 g, 4.3 mmol) in ACN/HCl (5 mL, 20:1) were added, and the mixture was vigorously stirred at 45 °C for an additional 3 h. The reaction mixture was filtered through Celite, and the solvent was removed *in vacuo*. The resultant solid was redissolved in pyridine (40 mL), and acetic anhydride (3.62 mL, 38 mmol) and DMAP (5 mg) were The reaction mixture was stirred at room temperature for 16 h, and then the added. solvent was removed in vacuo. The resultant syrup was dissolved in ethyl acetate (100 mL) and washed with brine (100 mL). Silica gel column chromatography eluting with ethyl acetate ($R_f = 0.3$) gave a mixture of the two epimers ((4S):(4R) 3:1) as a white solid (2.01 g, 40%). Recrystallization from toluene/petroleum ether (1:1) gave the pure (4S)isomer, **3**, as a white solid (1.35 g, 27%). ¹H NMR (CDCl₃, 400 MHz) δ 1.98 (s, 3H, CH₃), 2.03 (s, 3H, CH₃), 2.04 (s, 3H, CH₃), 2.04 (s, 3H, CH₃), 2.06 (s, 3H, CH₃), 2.14 (s, 3H, CH₃), 2.34 (dd, 1H, *J*_{3,3} = 14.1 Hz, *J*_{3,4} = 9.0 Hz, H-3), 2.59 (dd, 1H, *J*_{3,3} = 14.0 Hz, *J*_{3,4} = 3.9 Hz, H-3), 3.98 (dd, 1H, $J_{8,9} = 5.7$ Hz, $J_{9,9} = 12.5$ Hz, H-9), 4.26 (dd, 1H, $J_{8,9} = 3.1$ Hz, $J_{9,9} = 12.5$ Hz, H-9), 4.49 (ddd, 1H, $J_{4,5} = 1.5$ Hz, $J_{5,6} = 10.3$ Hz, $J_{NH,5} = 10.4$ Hz, H-5), 5.02 (ddd, $1H_{J_{7,8}} = 8.1 Hz, J_{8,9} = 3.1 Hz, J_{8,9} 5.7 = Hz, H-8$), 5.14 (ddd, $1H_{J_{3,4}} = 4.1$ Hz, *J*_{3,4} = 8.9 Hz, *J*_{4,5} = 1.5 Hz, H-4), 5.21 (dd, 1H, *J*_{5,6} = 10.3 Hz, *J*_{6,7} = 2.1 Hz, H-6), 5.35 (dd, 1H, $J_{6,7} = 2.1$ Hz, $J_{7,8} = 8.1$ Hz, H-7), 5.56 (d, 1H, $J_{NH,5} = 8.7$ Hz, NH), 5.57 (d, 1H, $J_{1',1'} = 1.0$ Hz, H-1'), 6.15 (d, 1H, $J_{1',1'} = 1.0$ Hz, H-1'). ESI-MS (+) m/z 554 (M + Na⁺).

Methyl 5-Acetamido-4,6,7,8,9-pentaacetoxy-2-dibenzylphosphorylnonanoate (4)

Compound **3** (300 mg, 0.56 mmol) was dissolved in methylene chloride (30 mL), and O_3 was bubbled through the solution at -78 °C until a blue color persisted. Excess O_3 was

purged by bubbling argon through the solution at -78 °C. A solution of sodium borohydride (68 mg, 1.8 mmol) in ethanol (30 mL) was then added. The mixture was allowed to stand at (a) -78 °C for 24 h or (b) 25 °C for 30 min. After removal of the solvent *in vacuo*, the solid was redissolved in ethyl acetate, which was extracted with brine and dried over anhydrous Na₂SO₄. After removal of the solvent *in vacuo*, the reduced product was used in the next step without further purification. Dibenzyl N,Ndiethylphosphoramidite (600 μ L, 2 mmol) was added to a solution of the reduced product (400 mg, 1.3 mmol) and 1,2,4-triazole (40 mg, 0.59 mmol) in CH₂Cl₂ (10 mL) and stirred at room temperature for 16 h. After removal of the solvent *in vacuo* the reaction mixture was redissolved in diethyl ether (50 mL). The solution was then cooled to -78 °C and stirred for 30 min after 1 mL of 30% H₂O₂ was added. The organic layer was then washed with saturated $Na_2S_2O_3$ and dried over Na_2SO_4 . After removal of the solvent *in vacuo*, the resultant syrup was washed with petroleum ether and purified using silica gel column chromatography (ethyl acetate, $R_f = 0.2$). Compound 4 was obtained as colorless syrup and was found to be comprised of a 4.5:1 mixture of (2S)-4:(2R)-4 using condition a or a 1.2:1 mixture of (2S)-4:(2R)-4 using condition b. ¹H NMR (CDCl₃, 400 MHz) δ 1.92 (s, 3H, (2S)-CH₃), 1.93 (s, 3H, (2R)-CH₃), 1.98 (s, 3H, (2S)-CH₃), 2.00 (s, 3H, (2R)-CH₃), 2.019 (s, 3H, CH₃), 2.023 (s, 3H, CH₃), 2.031 (s, 3H, CH₃), 2.077 (s, 3H, (2R)-CH₃), 2.092 (s, 3H, (2S)-CH₃), 2.1 (m, 2H, H-3), 3.96 (dd, 1H, $J_{8,9a} = 5.7$ Hz, $J_{9a,9b} = 12.4$ Hz, (2S)-H-9a), 4.06 (dd, 1H, $J_{8,9a} = 6.6$ Hz, $J_{9a,9b} = 6.7$ Hz, (2R)-H-9a), 4.18 (dd, 1H, $J_{8,9b} = 2.5$ Hz, $J_{9a,9b} = 6.7$ Hz, (2R)-H-9a), 4.18 (dd, 1H, $J_{8,9b} = 2.5$ Hz, $J_{9a,9b} = 2.5$ Hz, J6.7 Hz, (2*R*)-H-9b), 4.25 (dd, 1H, $J_{8.9b} = 2.8$ Hz, $J_{9a.9b} = 12.4$ Hz, (2*S*)-H-9b), 4.46 (ddd, 1H, $J_{4,5} = 1.5$ Hz, $J_{5,6} = 10.4$ Hz, $J_{NH,5} = 10.5$ Hz, (2S)-H-5), 4.60 (ddd, 1H, $J_{4,5} = 1.5$

Hz, $J_{5,6} = 10.4$ Hz, $J_{NH,5} = 10.5$ Hz, (2R)-H-5), 4.88 (ddd, 1H, $J_{3,4} = 4.6$ Hz, $J_{3,4} = 8.2$ Hz, $J_{4,5} = 1.5$ Hz, H-4), 5.00 (ddd, 1H, $J_{7,8} = 8.1$ Hz, $J_{8,9a} = 5.7$ Hz, $J_{8,9b} = 3.0$ Hz, H-8), 5.05–5.17 (m, 5H, Bn-CH₂ and H-2), 5.22 (dd, 1H, $J_{5,6} = 10.4$ Hz, $J_{6,7} = 1.8$ Hz, (2S)-H-6), 5.25 (dd, 1H, $J_{5,6} = 10.4$ Hz, $J_{6,7} = 1.8$ Hz, (2R)-H-6), 5.33 (dd, 1H, $J_{6,7} = 1.8$ Hz, $J_{7,8} = 8.1$ Hz, (2S)-H-7), 5.37 (dd, 1H, $J_{6,7} = 1.8$ Hz, $J_{7,8} = 8.1$ Hz, (2R)-H-7), 5.65 (d, 1H, $J_{NH,5} = 10.5$ Hz, (2R)-NH), 5.70 (d, 1H, $J_{NH,5} = 10.5$ Hz, (2S)-NH), 7.34 (m, 10H, Bn). ESI-MS (+) m/z 818 (M + Na⁺).

5-Acetamido-4,6,7,8,9-pentahydroxy-2-phosphorylnonanoic Acid

Ditriethylammonium Salt (1)

To a solution of **4** (80 mg, 0.10 mmol) in methanol (20 mL) was added Pd/C (10%, 30 mg), and the mixture was stirred under H₂ (1 atm) for 1 h. After filtration through Celite and removal of the solvent *in vacuo*, the resulting solid was dissolved in 1:1 MeOH/H₂O (20 mL) containing 10% triethylamine (TEA) and was allowed to stand at -20 °C for 14 h. After removal of the methanol*in vacuo*, the remaining aqueous solution was diluted with distilled H₂O and lyophilized to dryness. The solid was then purified by passage through a Bio-Gel P-2 column (2.5 cm × 44 cm) eluting with distilled water (0.2 mL min⁻¹). The fractions containing inhibitor **1**, as analyzed by negative ESI-MS, were lyophilized twice with distilled water to give **1** as a white solid (25 mg, 42%). The ratio of (2*S*)-**1**:(2*R*)-**1** reflected the ratio of (2*S*)-**4**:(2*R*)-**4** used in the reaction, as determined by ³¹P NMR spectroscopy. Due to the presence of many overlapping signals, ¹H and¹³C NMR data are only given for the major isomer (2*S*)-**1** obtained from deprotection of the **4**.5:1

mixture of (2*S*)-4:(2*R*)-4. ¹H NMR ((2*S*)-1, D₂O, 400 MHz) δ 1.29 (t, 2H, TEA CH₃), 1.68 (ddd, 1H, $J_{3a,3b} = 11.8$ Hz, $J_{3a,2} = 11.0$ Hz, H-3a), 1.94 (dd, 1H, $J_{3a,3b} = 12.1$ Hz, $J_{3b,4} = 11.0$ Hz, H-3b), 2.07 (s, 3H, CH₃), 3.21 (q, 2H, TEA CH₂) 3.48 (dd, 1H, $J_{7,8} = 8.9$ Hz, H-7), 3.65 (dd, 1H, $J_{8,9a} = 6.4$ Hz, $J_{9a,9b} = 11.8$ Hz, H-9a), 3.79 (ddd, 1H, $J_{7,8} = 8.9$ Hz, $J_{8,9a} = 6.3$ Hz, $J_{8,9b} = 2.7$ Hz, H-8), 3.85 (dd, 1H, $J_{8,9b} = 2.6$ Hz, $J_{9a,9b} = 11.8$ Hz, H-9b), 3.94 (dd, 1H, $J_{5,6} = 10.1$ Hz, H-5), 4.01 (dd, 1H, $J_{5,6} = 10.0$ Hz, H-6), 4.37 (dd, 1H, $J_{3,4} = 11$ Hz, H-4), 4.54 (ddd, 1H, $J_{2,3} = 2.8$ Hz, $J_{2,3} = 10$ Hz, $J_{2,P} = 10$ Hz, H-2). ¹³C NMR ((2*S*)-1, MeOD, 100 MHz) δ 9.3 (TEA), 22.6 (CH₃), 40.3 (C-3), 47.5 (TEA), 56.4 (C-5), 65.4 (C-9), 65.9 (C-4), 69.9 (C-6), 71.8 (C-7), 72.5 (C-8), 74.8 (C-2), 174.6 (C=O), 180.2 (O=C-OH). ³¹P NMR (D₂O, pD 7, 162 MHz) δ 2.30 ((2*R*)-1), 2.75((2*S*)-1). HRMS calculated for C₁₁H₂₁NO₁₂P (M – H⁺) 390.0801, found 390.0806.

2.2.3 Measurement of Inhibition Kinetics

Inhibition kinetics were measured using a slight modification of a previously reported continuous coupled assay for phosphate (Webb, 1992, Chou *et al.*, 2005). His-tagged NeuB was generated and purified as described previously (Gunawan et al., 2005). A cuvette containing Tris-HCl buffer (pH 7.0, 100 mM), MnCl₂ (1 mM), PEP (variable, 50 μ M to 1 mM), His-tagged NeuB (5 μ g), purine nucleoside phosphorylase (bacterial, Sigma, 5 units, previously buffer exchanged to 20 mM Tris-HCl, pH 7.0), 2-amino-6-mercapto-7-methylpurine ribonucleoside (200 μ M), and inhibitor **1** (variable, 0–20 μ M of the 4.5:1 mixture of (2*S*)-1:(2*R*)-1) was preincubated for 20 min at 37 °C. The enzymatic reaction was initiated by addition of ManNAc (10 mM). Rates were measured by monitoring the

increase of absorption at 360 nm ($\epsilon = 11000 \text{ M}^{-1} \text{ cm}^{-1}$). Kinetic parameters were determined by fitting initial velocities to the Michaelis–Menten equation using GraFit 5.0 (Erithacus software).

2.2.4 Cloning, Overexpression, and Purification of NeuB for Crystallization

All molecular biology procedures were performed as described previously (Gunawan et al., 2005). Briefly, the untagged NeuB enzyme was cloned into the pCWori+ vector and subsequently transformed into the electrocompetent *E. coli* cell (BL21 λ DE3; Novagen) for expression. Overexpression of NeuB was carried out by induction with 0.5 mM isopropyl β -D-galactopyranoside (IPTG) at OD₆₀₀ ~ 0.6 with overnight shaking at 20 °C. Cells were harvested by centrifuging at 5,000 rpm for 15 min, resuspended, and lysed at 20,000 psi using a high-pressure homogenizer (Avestin) in the presence of EDTA-free protease inhibitor cocktail (Roche Applied Science). The lysate was subsequently centrifuged at 40,000 rpm for 35 min, and the supernatant containing the target protein was purified by a series of chromatographic procedures including ion-exchange and gel filtration steps. Purified NeuB enzyme was concentrated to 10 mg/mL and used for crystallization.

2.2.5 Crystallization, Data Collection, and Structure Refinement

Purified NeuB enzyme was crystallized at 18 °C in the presence of 10 mM $MnCl_2$ and 1.50–1.55 M malic acid (pH 6.2) using the hanging-drop vapor diffusion technique and subsequently soaked in 2 M sodium phosphate (pH 6.2) for 24 h with 10 mM $MnCl_2$ and 3 mM of the 4.5:1 mixture of (2*S*)-1:(2*R*)-1. The soaked crystal was

transferred into the mother liquor containing 25% ethylene glycol for 10 s and frozen in liquid nitrogen prior to data collection. X-ray diffraction data were collected at 100 K under a nitrogen stream at the beamline 4.2.2 of the Advanced Light Source (Berkeley, CA) coupled to a NOIR-1 CCD detector. Collected data were processed by MOSFLM and SCALA (Leslie, 1992, Potterton *et al.*, 2003). The inhibitor **1** bound NeuB crystal belongs to the space group P2₁2₁2 with unit cell dimensions a = 58.62, b = 75.74, and c = 77.36 Å and contains one molecule in the asymmetric unit. Scaled data were directly used for the structure refinement by REFMAC5 (Murshudov *et al.*, 1997) with the coordinate of NeuB as a starting model (PDB accession code: 1XUZ). The required parameter file for inhibitor **1** was generated from the Dundee PRODRG2 server (Schuttelkopf & van Aalten, 2004), and all structural figures were produced using PyMOL (DeLano, 2002). Statistics for data collection and refinement are summarized in Table 2.1.

	NeuB+Inhibitor 1^{\dagger}						
Data Collection							
X-ray source	ALS 4.2.2						
wavelength (Å)	0.9790						
resolution $(\text{\AA})^a$	40-1.75 (1.84-1.75)						
space group	$P2_{1}2_{1}2$						
a (Å)	58.62						
b (Å)	75.74						
c (Å)	77.36						
α, β, γ (°)	90, 90, 90						
$R_{ m sym} \left(\%\right)^a$	10.5 (46.1)						
$I/\sigma(I)^a$	12.4 (2.2)						
completeness $(\%)^a$	99.3 (97.0)						
unique reflections ^{<i>a</i>}	35,158 (4926)						
redundancy ^a	4.0 (3.6)						
Refinement Statistics							
average B-factors ($Å^2$)							
protein	9.0						
ligand	13.8						
water	18.4						
ramachandran statistics							
favored regions (%)	98.9						
allowed regions (%)	1.1						
R_{work} (%)	16.3						
$R_{\rm free}$ (%)	19.7						
r.m.s. ^b bonds (Å)	0.011						
r.m.s. angles (°)	1.202						
^{<i>a</i>} Values in parentheses represent the highest resolution shell							
^{<i>b</i>} r.m.s., root mean square							

Table 2.1: Data Collection and Refinement Statistics.

[†] X-ray coordinates have been deposited in the Protein Data Bank (http://www.pdb.org, accession code 2WQP).

2.3 Results and Discussion

2.3.1 Synthesis of Inhibitor 1

The overall strategy used in the synthesis of inhibitor **1** involved the addition of a three-carbon masked pyruvate unit to the peracetylated open chain form of ManNAc, 2 (Figure 2.2). The aldehyde of ManNAc was first protected using hydroxylamine, and the exposed hydroxyl groups were peracetylated using acetic anhydride. Ozonolysis was used to unmask the aldehyde functionality and gave compound 2 in three steps, as reported previously (Weitz & Bednarski, 1989). An indium-mediated addition of methyl bromomethylacrylate, followed by acetylation with acetic anhydride, generated compound **3** as a 3:1 ratio of isomers at C-4. Column chromatography, followed by recrystallization, gave the major isomer of **3** bearing the required (S)-configuration at C-4. Previous studies on indium-mediated couplings of closely related compounds also show this selectivity and demonstrate that the major isomer bears a *syn* relationship between the newly formed hydroxyl group and the acetamido group, consistent with a chelate-controlled delivery (Paquette et al., 1997, Vorwerk & Vasella, 1998). Ultimately, crystallographic analysis of the complex between the final inhibitor 1 and sialic acid synthase confirmed this stereochemical outcome (vide infra).



Figure 2.2: The synthetic route used in the preparation of inhibitor 1.

Treatment of compound **3** with ozone exposed the ketone functionality at C-2, and reduction with sodium borohydride followed by phosphorylation gave compound **4** as a mixture of stereoisomers. When the reduction was carried out at -78 °C, a 4.5:1 mixture of (2*S*)-4:(2*R*)-4 was obtained, and when the reduction was carried out at 25 °C, a 1.2:1 mixture of (2*S*)-4:(2*R*)-4 was obtained. These isomers were not separable by conventional silica gel chromatography and were therefore carried on as a mixture. Deprotection of compound **4** was achieved via hydrogenolysis of the benzyl phosphate groups, followed by mild ester hydrolysis using triethylamine in MeOH/H₂O at -20 °C. Size exclusion chromatography gave pure inhibitor **1** as either a 4.5:1 mixture of (2*S*)-1:(2*R*)-1 or a 1.2:1 mixture of (2*S*)-1:(2*R*)-1, depending on the reduction conditions used to prepare compound **4**. The assignment of configuration at C-2 in compounds **4** and **1** was made by first determining which of the two isomers of **1** is a better inhibitor and then by analyzing the structure of the inhibited enzyme (vide infra).

2.3.2 Kinetic Evaluation of Inhibitor 1

Kinetic constants for the inhibition of the *N. menigitidis* sialic acid synthase by compound **1** were obtained using a continuous coupled assay for phosphate release (Webb, 1992, Chou et al., 2005). Initial studies were performed on the 4.5:1 mixture of (2*S*)-**1**:(2*R*)-**1** and indicated that compound **1** acted as a strong and slow binding inhibitor. Therefore, prior to each kinetic analysis, the enzyme was preincubated in the presence of the inhibitor (variable amounts), PEP (variable amounts), and Mn^{2+} (1 mM) for 20 min to ensure that binding equilibration had occurred. The reactions were then initiated by the

addition of a saturating amount of ManNAc (10 mM). Compound **1** was found to act as a competitive inhibitor against PEP with an apparent K_i value of $3.1 \pm 0.1 \mu$ M (Figure 2.3, 2.4). Identical kinetic runs using the 1.2:1 mixture of (2*S*)-1:(2*R*)-1 (containing 45% (2*R*)-1 instead of 18%) required an approximately 2.5-fold lower total inhibitor concentration in order to bring about a comparable reduction of rate (Table 2.2). This indicates that the minor (2*R*)-isomer is responsible for at least 80% of the inhibition. The fact that the value of K_i for compound 1 is significantly lower than the value of K_m for PEP (157 μ M under identical conditions) supports the notion that the inhibitor serves as a mimic of the tetrahedral intermediate presumed to form in the synthase reaction. Furthermore, the fact that one isomer binds more tightly than the other suggested that it would be possible to characterize a complex containing only this isomer.



Figure 2.3: Inhibition kinetics of inhibitor 1. (2S)-1:(2R)-1 = 4.5:1.



Figure 2.4: Replot data from Lineweaver-Burk plots for the determination of K_i of **inhibitor 1**. $K_i = 3.1 \pm 0.1 \ \mu M ((2S) - 1:(2R) - 1 = 4.5:1).$

Table 2.2:	Comparison	of	inhibition	by	4.5:1	mixture	of	(2 <i>S</i>)-1:(2 <i>R</i>)-1	and	1.2:1
mixture of	(2S)-1:(2R)-1.									

Inhibitor conc. (total, μ M)	Ratio of (2 <i>S</i>)-1:(2 <i>R</i>)-1	Initial velocity $(\mu M \min^{-1})^{a}$
10	4.5:1	2.80
10	1.2:1	1.80
5	4.5:1	3.29
2	1.2:1	3.34

^a Initial velocity measurements were made using the coupled assay described in the Experimental Procedures Section with $[PEP] = 250 \mu M$, [ManNAc] = 10 mM, and [NeuB]= 45 nM.

2.3.3 Crystallographic Analysis of the Complex between Inhibitor 1 and Sialic Acid Synthase

Previous crystallographic studies showed that the N. meningitidis sialic acid synthase, NeuB, exists as a domain-swapped homodimer with 2-fold symmetry coincident with that of the 2-fold symmetry axis of the orthorhombic crystal form (Figure 2.5A, B) (Gunawan et al., 2005). Each monomer consists of an N-terminal $(\alpha/\beta)_8$ barrel (TIM barrel) and a "pretzel-shaped" C-terminal domain that bears high sequence identity and structural similarity to the ice binding type III antifreeze protein. The C-terminal domain of one subunit caps the TIM barrel of the opposing subunit and is intimately involved in hydrogenbonding interactions in the active site. This earlier structure of NeuB was solved to 2.2 Å resolution as a complex with N-acetylmannositol (substrate reduced at C-1), PEP, and Mn²⁺, providing an excellent model of the Michaelis complex (Figure 2.5C). The metal ion was coordinated in an octahedral manner with His-215, His-236, the phosphate of PEP, and a bound water (W_{eq}) serving as the equatorial ligands and with the C-1 hydroxyl of Nacetylmannositol and a second bound water (Wax) serving as the axial ligands. The observation of direct coordination between the hydroxyl of N-acetylmannositol and the metal ion was consistent with the proposed role of the metal in activating the aldehyde of ManNAc in the normal reaction mechanism. This structure also showed that the *si* face of PEP faces the C-1 of the N-acetylmannositol, consistent with the reported stereochemical outcome of the synthase reaction (Sundaram et al., 2004). The carboxylate of PEP was twisted by 30° with respect to the alkene functionality, presumably to reduce conjugation and increase the nucleophilicity of the alkene.



Figure 2.5: Overall structure and substrate binding site of NeuB (PDB accession code 1XUZ).

(A) Ribbon representation of the NeuB monomer structure showing the TIM barrel and antifreeze-like domains. (B) Domain-swapped homodimeric structure of NeuB with each monomer colored red or green. Bound substrates which delineate the active site (*N*-acetylmannositol, PEP) are shown in CPK colored stick representations. The active site is formed at the interface of two monomers as highlighted by the black dotted box. (C) Active site of NeuB focusing on the essential metal coordination. The metal ion is shown as a magenta sphere (relative radius reduced for clarity), and histidine residues (His-215, His-236) and bound substrates are shown as a CPK colored stick model (carbon, yellow; oxygens, red; nitrogens, blue; and phosphorus, orange). Water molecules (W_{ax}, W_{eq}) are shown as blue spheres. Hydrogen bonds are represented by black dotted lines.

In order to obtain a complex with inhibitor 1, NeuB was first crystallized in the presence of 10 mM MnCl₂ and 1.50-1.55 M malic acid (pH 6.2) to generate the malic acid/Mn²⁺ complex described previously (Gunawan et al., 2005). The NeuB crystals were then soaked in 2 M phosphate buffer (pH 6.2) containing 10 mM MnCl₂ and 3 mM of the 4.5:1 mixture of (2S)-1:(2R)-1. This resulted in the formation of a NeuB·inhibitor $1 \cdot Mn^{2+}$ complex that could be structurally characterized to a resolution of 1.75 Å with excellent stereochemical quality and $R_{\text{work}}/R_{\text{free}}$ values (0.163/0.197) (Table 2.1). The bound inhibitor 1 was clearly visible at 3.5σ in the $F_o - F_c$ maps (before any additional refinement) and was judged to be fully occupied within the active site in refined $2F_{o}-F_{c}$ maps (Figure 2.6). Inspection of the structure clearly shows that only a single stereoisomer, (2R)-1, is present, indicating that the enzyme had selected this less prevalent but tighter binding isomer from the incubation mixture. The overall structure was very similar to that of NeuB·N-acetylmannositol·PEP·Mn²⁺, with a root-mean-square deviation (rmsd) of 0.16 Å on all 345 C α atoms. The phosphate and carboxylate groups of 1 occupy similar positions and engage in analogous hydrogen-bonding interactions to the corresponding groups of PEP and N-acetylmannositol in the previous structure (Figure 2.7A). The phosphate group of 1 establishes a number of hydrogen bonds to various conserved residues including Ser-132, Ser-154, Ser-213, and Lys-129, as well as Asn-184 in the active site (Figure 2.7B). The carboxylate of **1** interacts electrostatically with the conserved lysine residues (Lys-53, Lys-129) as well as via a hydrogen bond with Thr-110.



Figure 2.6: Observed electron density of inhibitor (2*R*)-1.

Stereoview of the observed electron density of the NeuB-bound inhibitor **1** in (A) the initial $F_o - F_c$ map contoured at 3.5 σ and (B) the refined $2F_o - F_c$ map contoured at 1.5 σ . The (*R*) configuration of inhibitor **1** is highlighted with an arrow at the C-2 position. Carbon atoms are represented in green. Non-carbon atoms are colored according to atom type (nitrogen, blue; oxygen, red; and phosphorus, orange).



Figure 2.7: The active site of NeuB complexed with inhibitor (2*R*)-1.

(A) Comparison of active sites between the NeuB inhibitor $1 \cdot Mn^{2+}$ and the NeuB $\cdot N$ -acetylmannositol·PEP·Mn²⁺ structures. Interacting amino acid residues are shown in CPK coloring with carbons in gray. Carbon atoms in the inhibitor 1, *N*-acetylmannositol, and PEP are displayed with green, yellow, and light blue, respectively. Lys-53 and Gln-55 have been omitted for clarity. (B) Active site of the NeuB inhibitor $1 \cdot Mn^{2+}$ complex. All interacting amino acid residues are shown in CPK with carbons in yellow from one monomer and in cyan from the adjacent monomer with black dotted lines representing hydrogen bonds. Carbon atoms in inhibitor 1 are displayed with green, and non-carbon atoms are colored according to atom type (nitrogen, blue; oxygen, red; and phosphorus, orange). Manganese ion and water molecules are represented as magenta and cyan spheres, respectively. Asn-184 has been omitted for clarity.

All hydroxyl groups of inhibitor **1** are also involved in the extensive hydrogen-bonding network through a series of conserved residues (Gln-55, Tyr-186, and Asp-247) and water molecules. The *N*-acetylamino group of inhibitor **1** also interacts with the enzyme via water-mediated contacts. One water molecule bridges between the carbonyl moiety of the *N*-acetylamino group and the side chain carboxylate of Glu-134. The other water molecule interacts with both the carbonyl moiety of the *N*-acetylamino group of inhibitor **1** and also establishes a direct contact to the conserved Arg-314' residue from the neighboring monomer in the homodimeric complex.

An analysis of the tetrahedral geometry at C-2 clearly indicates an (R)-configuration of the bound inhibitor and provides the basis for the assignment of stereochemistry of the tighter binding isomer. Assuming that this stereochemical preference reflects a resemblance to the tetrahedral intermediate, then the intermediate is also expected to bear an (R)-configuration at C-2. This would indicate that the reaction mechanism involves an attack of water onto the *si* face of the oxocarbenium ion intermediate (Figure 2.8).



Figure 2.8: Revised mechanism of the reaction catalyzed by sialic acid synthase. The proposed stereochemistry of the tetrahedral intermediate and the dual role played by the Mn^{2+} ion are outlined.

The (2*R*)-configuration orients the C-2 hydrogen of inhibitor **1** toward the Mn^{2+} ion and suggests that in the actual tetrahedral intermediate the C-2 hydroxyl may serve as a ligand for the metal. It also implies that the metal may play a dual role in catalysis, both as an electrostatic catalyst that activates the aldehyde of ManNAc and as a source of the activated water molecule that attacks the oxocarbenium ion intermediate (Figure 2.8). In the NeuB·*N*-acetylmannositol·PEP·Mn²⁺ structure, the equatorial metal-bound water molecule (W_{eq}) is positioned 2.8 Å away from the *si* face of the bound PEP (3.1 Å away from C-2 of PEP) and is a likely candidate to play the role of the nucleophile (Figure 2.5). Glu-25 and Glu-234 are hydrogen-bonded to W_{eq} and serve as reasonable candidates for the basic residue that deprotonates W_{eq} during its addition to the oxocarbenium ion intermediate. In the structure of NeuB·inhibitor **1**·Mn²⁺, the electron density corresponding to the Mn²⁺ ion indicates only partial occupancy (50%) based on resulting maps and temperature factors.

The partial occupancy of the metal cofactor indicates that the manganese ion binds weakly to the NeuB inhibitor 1 complex. This notion is further supported by the determination of NeuB inhibitor 1 structures devoid of bound metal cofactor that were obtained during various soaking trials. The manganese ion of the NeuB·inhibitor $1 \cdot Mn^{2+}$ complex was coordinated in the active site with a significantly distorted octahedral arrangement, whereas the previously reported NeuB·N-acetylmannositol·PEP·Mn²⁺ complex displayed the much more regular octahedral geometry typical of Mn²⁺ binding coordination spheres. The metal-ligand interactions are largely maintained in the NeuB·inhibitor 1·Mn²⁺ structure, except that W_{eq} has moved 0.6 Å away from the bound inhibitor and the distance between the metal and W_{ax} has increased by 0.8 Å (Figure 2.7B). In addition, W_{ax} loses the contact with the carboxylate of Glu-234 and now interacts with Ser-213 and main chain carbonyls of Asp-214 and Glu-234, thereby moving ~25° from its position in the previous structure to create the distorted octahedral geometry. The partial occupancy and distorted geometry of the bound metal suggest that the binding of inhibitor 1 disturbs the coordination sphere and likely impairs binding somewhat. One explanation may lie in the fact that the C-2 hydrogen of the inhibitor is oriented toward the metal and thereby has displaced W_{eq} from its preferred position. In the normal reaction mechanism, the C-2 hydroxyl group of the tetrahedral intermediate would occupy this coordination site and no steric clash would result. An alternative explanation for the perturbed metal binding/geometry could be that a conformational change normally accompanies the formation of the tetrahedral intermediate but that soaking with the inhibitor is not sufficient to induce the same change in the solid state. In this event, the active site would not be in an optimal conformation to

bind both inhibitor **1** and the metal ion, and the metal binding could be impaired. Unfortunately, our attempts to obtain a structure by cocrystallization were unsuccessful.

2.4 Conclusions

The bacterial sialic acid synthases are most closely related to the mammalian sialic acid synthases and to other metal-dependent bacterial enzymes that catalyze the biosynthesis of structurally similar α -keto acids. These include pseudaminic acid synthase (NeuB shares 35% identity with C. jejuni enzyme) and N,N-diacetyllegionaminic acid synthase (NeuB shares 61% identity with the Legionella pneumophila enzyme) (Chou et al., 2005, Glaze *et al.*, 2008, Schoenhofen *et al.*, 2009). Sialic acid synthese is more distantly related to other a-keto acid synthases such as 2-keto-3-deoxy-D-manno-octulosonate-8phosphate synthase (KDO8PS) and 2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAH7PS) (Liang et al., 1998, Howe et al., 2003, Furdui et al., 2004). The latter enzymes do not share significant sequence identity with NeuB (<10%) and do not possess the unique C-terminal domain, yet are known to adopt a TIM barrel protein fold (Shumilin et al., 1999, Radaev et al., 2000). While all of the α-keto acid synthases utilize a C-O bond cleavage mechanism (DeLeo et al., 1973, Hedstrom & Abeles, 1988, Chou et al., 2005, Gunawan et al., 2005, Glaze et al., 2008), the requirement for a metal ion cofactor is not universal (Duewel & Woodard, 2000, Sau et al., 2004, Shulami et al., 2004).

Previous studies on the generation of mechanism-based inhibitors of the α -keto acid synthases have focused on the KDO8PS and DAH7PS enzymes (Asojo *et al.*, 2001, Wang *et al.*, 2001, Kaustov *et al.*, 2003, Walker & Parker, 2006). Much of that work centered on the preparation of amine-bearing derivatives that mimic the oxocarbenium ion intermediate thought to form during catalysis. One report described the synthesis of phosphonate-based analogues of the tetrahedral intermediates formed by these enzymes; however, kinetic studies were not presented (Grison et al., 2005). This report describes the first reported inhibitor of a sialic acid synthase, inhibitor 1, which is a 2-deoxy analogue of the putative tetrahedral intermediate. The compound was synthesized as a mixture of stereoisomers at C-2 in order to probe the preference of the active site in binding a given configuration. Inhibition studies and structural analysis confirm that the inhibitor bearing the (2R)configuration is bound more tightly than the other. This suggests that the tetrahedral intermediate also bears a (2R)-configuration and that the C-2 hydroxyl group is directed toward the active site metal ion. The mechanistic implications of this arrangement are that the metal serves not only to activate the carbonyl of ManNAc for attack by PEP in the first step of the reaction but also to deliver hydroxide to the oxocarbenium ion intermediate in the second step of the reaction (Figure 2.8). While stereochemical analyses based on the use of intermediate analogues often provide key insights into the mechanisms of enzymatic reactions, it must be mentioned that they can be misleading. An example of this can be found with the enzyme 5-enolpyruvylshikimate-3-phosphate synthase where a phosphonate-based inhibitor bearing the non-natural configuration at the transient tetrahedral center bound more tightly than the one with the natural configuration (Priestman *et al.*, 2005). While it is not possible to isolate the tetrahedral intermediate formed in the sialic acid synthase reaction and determine its stereochemistry directly, the preparation and analysis of additional analogues could reinforce the findings of this study. Toward that end, the syntheses of phosphonate-based analogues that retain the C-2 hydroxyl group are currently being pursued in these laboratories.

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<u>CHAPTER 3</u>: STRUCTURAL AND KINETIC CHARACTERIZATIONS OF THE POLYSIALIC ACID *O*-ACETYLTRANSFERASE OATWY FROM *NEISSERIA MENINGITIDIS*[¶]

3.1 Introduction

The bacterial pathogen *Neisseria meningitidis* is a major cause of life-threatening neuroinvasive meningitis in humans (Ryan *et al.*, 2004). In the United States, 75% of bacterial meningitis infections are caused by serogroup C, Y, or W-135 (Bilukha & Rosenstein, 2005). In particular, the proportion of meningococcal infection occurrences in the United States caused by the group Y meningococci has increased significantly from 2% during 1989–1991 to 37% during 1997–2002 (Bilukha & Rosenstein, 2005). Vaccines based on the capsular polysaccharide have been developed for groups A/C/Y/W-135 (Bilukha & Rosenstein, 2005), and the introduction of a group C conjugate vaccine has reduced the incidence and carriage of the C serogroup significantly (Maiden *et al.*, 2008). Although these vaccines are working, they do not yet provide complete protection from meningococcal disease (Stephens, 2007).

The capsular polysaccharides of *N. meningitidis* are classified into 13 distinct serogroups based on their chemical structures (Virji, 2009). The capsules of serogroup B and C are homopolymers composed of α -2,8- or α -2,9-linked sialic acid, respectively, whereas serogroup Y and W-135 are heteropolymers of an α -2,6-linked sialic acid on

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glucose (Y) or galactose (W-135) (Bhattacharjee *et al.*, 1975, Bhattacharjee *et al.*, 1976). *N. meningitidis* group B polysialic acid shares a biochemical epitope with the polysialylated form of the neural cell adhesion molecule of humans (Troy, 1992, Kleene & Schachner, 2004). Because of this molecular mimicry of the polysialic acid-neural cell adhesion molecule, the bacterial capsular polysaccharide is thus considered a major virulence factor of *N. meningitidis* (Moxon, 1990, Virji, 2009).

Serogroup C, Y, and W-135 of *N. meningitidis* modify their sialic acid capsules by *O*-acetylation of the sialic acid (Lemercinier & Jones, 1996). Sialic acid is acetylated at the C-7 or C-8 position hydroxyl group in serogroup C, whereas the C-7 or C-9 position is acetylated in serogroup W-135 and Y (Lemercinier & Jones, 1996). The *O*-acetylation of sialic acids is known to alter the physicochemical properties of the polysaccharide capsule (Klein & Roussel, 1998). In addition, there is growing evidence that *O*-acetylation of the polysaccharide enhances bacterial pathogenesis by masking the protective epitope in the polysaccharide (Frasa *et al.*, 1993, Bhasin et al., 1998, Kim & Slauch, 1999, Fusco et al., 2007). For these reasons, considerable effort has been expended to identify and characterize sialic acid *O*-acetyltransferases in pathogenic bacteria.

Recently, the sialic acid-specific *O*-acetyltransferases from group B *Streptococcus, Campylobacter jejuni, Escherichia coli* K1, and *N. meningitidis* serogroup C have been identified (Claus *et al.*, 2004, Lewis *et al.*, 2004, Houliston *et al.*, 2006, Lewis AL, 2006) with the latter two variants being the only ones to be characterized biochemically (Deszo *et al.*, 2005, Bergfeld *et al.*, 2007, Bergfeld *et al.*, 2009). These studies showed that bacterial sialic acid-specific *O*-acetyltransferases utilize an acetyl-CoA

cofactor as a donor for the acetylation of their capsular sialic acid acceptor substrates (Figure 3.1) and identified essential amino acid residues for potential catalytic roles in activity (Bergfeld et al., 2007, Bergfeld et al., 2009). Although the gene encoding the capsule-specific *O*-acetyltransferase in *N. meningitidis* serogroup Y (known as OatWY) has been identified, biochemical characterization of the enzyme has not yet been reported. Furthermore, the lack of structural information on a sialic acid *O*-acetyltransferase from any bacterial species has hampered our ability to further understand the mode of substrate binding, specificity, and catalytic mechanism of this important sialic acid-modifying family.

Here we report the first kinetic and structural analysis of polysialic acid *O*-acetyltransferase OatWY from *N. meningitidis* serogroup Y in complex with either CoA, acetyl-CoA, or *S*-(2-oxopropyl)-CoA, which is a nonhydrolyzable acetyl-CoA substrate analogue. Collectively, this study significantly contributes to our understanding of bacterial polysialic acid *O*-acetyltransferases, providing valuable insight into how capsular polysaccharide is acetylated in pathogenic bacteria.


Figure 3.1: Reaction scheme of the OatWY-catalyzed O-acetyltransferase.

Although acetylation of both the O-7 and O-9 hydroxyl group of the *N*. *meningitidis* serogroup Y polysialic acid has been implied through NMR analysis of the corresponding bacterial capsule (Lemercinier & Jones, 1996), for simplicity only the O-9 transfer is shown here.

3.2 Experimental Procedures

3.2.1 Cloning of the Polysialic Acid O-Acetyltransferase OatWY from N. meningitidis

Y

The *oatWY* gene (GenBankTM accession number Y13969) coding for the polysialic acid *O*-acetyltransferase was directly amplified by PCR from genomic DNA of the *N*.

meningitidis serogroup Y using the *Pwo* DNA polymerase (Stratagene) with the forward primer OATWY-F (5'-CCGACG<u>CATATG</u>GGAACTCACATGTATTCTGAACAGGGAATTAATAATAC-3'), which introduced an NdeI site (underlined) in the 5' end, and the reverse primer OATWY-R (5'-

3.2.2 Cloning of OatWY Mutants by Site-directed Mutagenesis

Site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's guideline with the following OATWY-H121A-F (5'primers. GAAATACTGATATGGCTCCAATTTATTCTTTAGAAAATGGCGAACG-3') and OATWY-H121A-R (5'-CGTTCGCCATTTTCTAAAGAATAAATTGGAGCCATATCAGTATTTC-3') were used to the H121A OATWY-W145A-F (5'generate mutant. CGGTAATCACGTGGCGCTTGGCCGCAATGTTAC-3') and OATWY-W145A-R (5'-

GTAACATTGCGGCCAAGCGCCACGTGATTACCG-3') were used to generate the W145A OATWY-Y171A-F (5'-Furthermore. mutant enzyme. GTTGTAGGTTCTCATACTGTGCTAGCTAAAAGTTTTAAAGAACC-3') and OATWY-Y171A-R (5'-GGTTCTTTAAAACTTTTAGCTAGCACAGTATGAGAACCTACAAC-3') were used to generate the Y171A mutant. All mutations were confirmed by DNA coding the mutant enzymes transformed sequencing. and genes into were electrocompetent E. coli cells as described above.

3.2.3 Expression and Purification of OatWY

The transformed cells were incubated overnight at 37 °C with shaking at 225 rpm in the presence of 50 µg/mL kanamycin. The cell cultures were grown until OD₆₀₀ reached ~0.6 and were induced by the addition of 1 mM isopropyl β -D-thiogalactopyranoside at 20 °C with further overnight incubation. Cells were harvested by centrifugation at 5,000 rpm for 15 min and then either used immediately or frozen at -80 °C for storage. The cells were resuspended in lysis buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 5 mM imidazole) and lysed in the presence of an EDTA-free protease inhibitor mixture tablet (Roche Applied Science) at 20,000 p.s.i. using a high pressure homogenizer (Avestin). The lysate was centrifuged at 40,000 rpm for 40 min, and the resulting supernatant was loaded onto a pre-equilibrated HiTrap Chelating HP column (GE Healthcare) charged with NiCl₂. The column was washed with buffer containing 100 mM imidazole and eluted with 100 mM EDTA in the buffer described above. All eluting fractions were monitored by SDS-PAGE. Fractions containing the OatWY protein were collected and dialyzed against 20 mM MES, pH 6.0, 100 mM NaCl with thrombin at 4 °C overnight. The dialyzed proteins were further purified on a Mono S cation exchange column (GE Healthcare) using a linear gradient of 100 mM to 1M NaCl in 20 mM MES, pH 6.0, and concentrated using an Amicon concentrator (Millipore). The molar mass of the protein was monitored by matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

3.2.4 In Vitro Activity Assay of OatWY

Activities of the wild type OatWY enzyme and mutants were determined in a spectrophotometric assay using the procedures reported previously (Alpers *et al.*, 1965). The common reaction mixture (200 µL final volume) was composed of 20 mM Tris-HCl, pH 7.5, 25 mM EDTA, 4 mM 5,5'-dithiobis(2-nitrobenzoic acid), 5.5 mg/mL Y meningococcal specific polysaccharide ($[\rightarrow 6)$ -Glc-($\alpha 1\rightarrow 4$)-Sia-($\alpha 2\rightarrow]_n$) in its deacetylated form, 1 mM acetyl-CoA, and the enzymatic reaction was initiated by adding 34 µM purified OatWY enzyme at 25 °C. The change of absorbance per min was monitored continuously at 412 nm using a Cary 300 spectrophotometer (Varian) regulated by a temperature controller. For further investigations regarding acceptor specificity, CMP-Neu5Ac, Neu5Ac, glucose, colominic acid (Sigma), and [Glc-($\alpha 1\rightarrow 4$)-Sia] disaccharide were standardized to an equal concentration of sialic acid (5 mM), ensuring equal saturation of the acceptor site by these various sialylated substrates. In addition, one assay mixture contained 1 mM *S*-(2-oxopropyl)-CoA instead of acetyl-CoA in the presence of the Y polysaccharide to investigate its reactivity toward the OatWY enzyme.

3.2.5 Kinetic Characterization

Kinetic parameters for acceptor (deacetylated Y polysaccharide) and donor (acetyl-CoA) were determined with OatWY native enzyme as follows. Assays were performed at 25 °C in a total volume of 200 μ L containing acceptor and donor with enzyme (34 μ M) in 20 mM Tris-HCl, pH 7.5, 25 mM EDTA, plus 4 mM 5,5'-dithiobis(2-nitrobenzoic acid) holding one substrate at a saturating concentration, although the other substrate concentration was varied. For donor kinetics, a range of different concentrations of acetyl-CoA (0.025–0.8 mM) was used, at a saturating concentration of the Y polysaccharide ([\rightarrow 6)-Glc-(α 1 \rightarrow 4)-Sia-(α 2 \rightarrow]_n, 5.5 mg/mL). For acceptor kinetics, varied concentrations of the Y polysaccharide (1.0–15.0 mg/mL) were used, at a saturating concentration of the data to the Michaelis-Menten equation using nonlinear regression analysis with GraFit 5.0 software (Erithacus Software).

3.2.6 Multiangle Light Scattering

Purified OatWY enzyme (5 mg/mL) was loaded onto a Superdex 200 10/300 GL gel filtration column (GE Healthcare), equilibrated with buffer (20 mM Tris, pH 7.5, 50 mM NaCl), and connected in line with miniDAWN multiangle light-scattering equipment coupled to an interferometric refractometer (Wyatt Technologies). Data analysis was done in real time using ASTRA (Wyatt Technologies), and molecular masses were calculated using the Debye fit method.

3.2.7 Synthesis of S-(2-Oxopropyl)-CoA

The synthesis of *S*-(2-oxopropyl)-CoA was performed according to the method described previously (Kim *et al.*, 2006). Briefly, 100 mg (0.13 mmol) of CoA trilithium salt (Sigma) was dissolved in 5 ml of water, followed by addition of dithiothreitol (11 mg, 0.07 mmol) and Li₂CO₃ (60 mg, 0.81 mmol) under argon atmosphere. To the resulting mixture was then added chloroacetone (5.3 mg, 0.06 mmol) in 12 ml of 95% ethanol. The reaction mixture was stirred at room temperature for 24 h, and then the solution was filtered and concentrated *in vacuo* affording a yellow oil. Reverse phase HPLC purification using Luna C18(2) column (Phenomenex), with HPLC grade acetonitrile, 0.1% trifluoroacetic acid, and HPLC grade water, 0.1% trifluoroacetic acid, using a 40–60% acetonitrile/water gradient over 60 min, yielded 30 mg (29%) of *S*-(2-oxopropyl)-CoA.

3.2.8 Deacetylation of Y Meningococcal Polysaccharide

The deacetylation of *N. meningitidis* serogroup Y polysaccharide was carried out according to previously published procedures (Bhattacharjee et al., 1975). Polysaccharide (70 mg) was dissolved in 0.1 M NaOH (30 mL), and the reaction mixture was stirred for 4 h at 30 °C. The solution was neutralized to pH 7.0 with acetic acid and then filtered through a 5-kDa molecular mass cutoff filter (Millipore). Flow-through was discarded, and the supernatant was concentrated to 2 mL of syrup. 1 mL of this syrup was lyophilized, and 1 mL was further used in the synthesis of 4-*O*- α -D-glucopyranosyl- β -*N*-acetylneuraminic acid.

3.2.9 Preparation of the [Glc- $(\alpha 1 \rightarrow 4)$ -Sia] Disaccharide

1 mL of de-*O*-acetylated Y polysaccharide was added to Amberlite IR-120 cation exchange resin (H^+ form) until pH 3.0 was achieved, and the reaction mixture was then incubated for 30 min at 100 °C. The resin was filtered off, and the reaction mixture was loaded onto Dowex® 1 × 2 ion exchange resin (Sigma) and eluted using a linear gradient of 0–1 M formic acid. Low resolution mass spectrometry showed the desired peaks in both positive and negative ion modes (M+1, 472 and M–1, 470). The resulting compound was characterized by proton NMR spectroscopy.

3.2.10 Crystallization and Data Collection

Crystals of OatWY were grown by the hanging drop vapor diffusion technique at 21 °C. The first condition that produced well ordered crystals contained 100 mM sodium acetate trihydrate, pH 4.6, 1.0 M ammonium phosphate monobasic, 100 mM lithium sulfate monohydrate and contained one molecule per asymmetric unit with unit cell dimensions of a = 199.635, b = 199.635, c = 199.635 Å in space group F4₁32. In the second condition, the reservoir solution contained 100 mM sodium acetate, pH 4.6, 2.25 M ammonium acetate. This OatWY crystal belonged to space group P222, with unit cell dimensions of a = 79.06, b = 94.83, c = 101.06 Å, and contained three molecules per asymmetric unit. The crystals were cryoprotected in mother liquor containing 25% ethylene glycol and directly plunged into liquid nitrogen prior to data collection. Three co-substrate structures were solved from apo-OatWY crystals (P222) soaked in either the presence of 1 mM coenzyme A, acetyl-CoA, or *S*-(2-oxopropyl)-CoA. The same reservoir solution as

described above was used, and the soaking times of either 4 h or overnight before flashfreezing in liquid nitrogen. X-ray diffraction data were collected at 100 K under a nitrogen stream using an in-house CuKα rotating anode x-ray generator coupled to a Mar345 detector or using synchrotron sources (beamlines 8.2.2 and 4.2.2 at the Advanced Light Source (Berkeley, CA) using an ADSC Q315 and NOIR-1 CCD detector, respectively, and the beamline 08ID-1 at the Canadian Light Source (Saskatoon, Saskatchewan, Canada) using a Marmosaic CCD225 detector). All diffraction data were processed and scaled by HKL or MOSFLM (Leslie, 1992, Øtwinowski Z., 1997).

3.2.11 Structure Determination, Refinement, and Modeling

The structure of OatWY was solved by the method of single isomorphous replacement with anomalous scattering. Crystals were soaked into the previously described reservoir solution containing the ethylene glycol cryoprotectant as well as the derivatizing agent 1 M NaI for 30 s and flash-frozen in liquid nitrogen. Positions of 11 iodide sites were found and refined by SHELX-C (Fortier, 1998). Phase calculation and solvent flattening were carried out by using SHELX-DE (Schneider & Sheldrick, 2002, Sheldrick, 2002), which resulted in an interpretable electron density map. Subsequent automatic model building by RESOLVE (Terwilliger, 2003) generated ~60% of the structure followed by further manual building using COOT (Emsley & Cowtan, 2004). Refinements were performed using REFMAC5 (Murshudov *et al.*, 1997) with exclusion of 5% of the reflections for the *R*_{free} calculation. Finally, Translation/Libration/Screw motion determination was incorporated into the procedure for further rounds of crystallographic

refinement (Painter & Merritt, 2006). All the ligand models, including CoA, acetyl-CoA, and S-(2-oxopropyl)-CoA, were generated using the PRODRG server (Schuttelkopf & van Aalten, 2004). We found two partially occupied molecules (0.7) of CoA and S-(2oxopropyl)-CoA, and three fully occupied molecules of acetyl-CoA at the interface of adjacent monomers of the P222 crystal form. The lack of occupancy of one interface of the homotrimeric subunits in the P222 crystal form may be due to the crystal packing and/or soaking method used for CoA incorporation (4 h for CoA or S-(2-oxopropyl)-CoA and overnight for acetyl-CoA, respectively). Final models were validated with MOLPROBITY (Davis et al., 2007). Modeling of Y meningococcal polysialic acid acceptor was performed using AutoDock Vina (Trott & Olson, 2010) with AutoDockTools (Sanner, 1999) and the necessary ligand file prepared using the PRODRG server (Schuttelkopf & van Aalten, 2004). A linear form of the polysialic acid, $[\rightarrow 6)$ -Glc- $(\alpha 1 \rightarrow 4)$ -Sia- $(\alpha 2 \rightarrow)_4$, was generated and tested for docking to the general area of potential acceptor binding between two adjoining monomers as suggested by our structural data. Subsequently, this roughly localized model $[\rightarrow 6)$ -Glc- $(\alpha 1 \rightarrow 4)$ -Sia- $(\alpha 2 \rightarrow)_4$ was utilized as the starting point in the docking procedure, which applied torsional rearrangements for the acceptor model to generate the lowest energy docking trials. Figures were generated with PyMOL (DeLano, 2002), and electrostatic surface calculations were carried out with the APBS plugin (Baker et al., 2001).

3.3 Results

3.3.1 Overall Architecture of OatWY

The crystal structure of the OatWY apoenzyme was solved by single isomorphous replacement with anomalous scattering utilizing phases from the sodium iodide soak. Cosubstrate structures of OatWY were determined using the native model in a molecular replacement procedure. The high resolution edge of the diffraction data varied from 1.90 to 2.35 Å. R_{work} values ranged from 17.8 to 20.1% and R_{free} values from 20.7 to 24.7% with full details of the data collection and refinement summarized in Tables 3.1. Of the two distinct crystal forms resulting from our crystallization, the structure derived from the crystal defined by space group P222 contained three molecules per asymmetric unit. The structure derived from the crystal with space group F4₁32 (one molecule per asymmetric unit) adopts a nearly identical trimeric arrangement around the 3-fold symmetry axis of the The crystallographic models derived from the two unique crystal forms of OatWY crystal. are highly similar and can be overlapped with a root mean square deviation of 0.72–0.88 Å on all 210 C α atoms. We believe the observed quaternary structure of OatWY as a homotrimer is physiologically relevant as supported by our multiangle light-scattering analysis in solution (Figure 3.2) and earlier work describing the catalytically essential homotrimerization of other acetyltransferases of the LBH family (Beaman et al., 1998, Wang et al., 2002, Lo Leggio et al., 2003).

	OatWY NaI	OatWY apo	OatWY apo
Data collection		AIS 8 2 2	AIS 8 2 2
Data conection	Cu Ka	ALS 8.2.2	ALS 8.2.2
Wavelength (Å)	1.5418	1.000	1.000
Resolution $(Å)^a$	40-2.7	40-1.95	40-2.20
	(2.80 - 2.70)	(2.02 - 1.95)	(2.28–2.20)
Space group	P222	F4 ₁ 32	P222
a (Å)	78.86	199.63	79.09
b (Å)	95.16	199.63	94.81
c (Å)	101.13	199.63	100.85
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90
$R_{\rm sym}$ (%) ^a	12.5 (50.3)	8.6 (45.3)	5.2 (26.9)
$I/\sigma(I)^a$	18.5 (3.6)	68.0 (10.8)	42.8 (6.5)
Completeness $(\%)^a$	100 (99.8)	100 (100)	100 (100)
Unique reflections ^{<i>a</i>}	40,415 (4058)	25,297 (2489)	39,235 (3848)
Redundancy ^a	7.7 (7.1)	46.9 (47.6)	8.1 (8.2)
Refinement			
Average B factor (λ^2)			
Protein		11.1	18.1
Ligand		11.1	10.1
Water		26.4	26.0
water		20.4	20.9
Ramachandran statistics			
Favored regions (%)		98.2	96.9
Additionally allowed regions (%)		1.8	3.1
Disallowed regions (%)		0.0	0.0
$R_{ m work}$ (%)		17.2	18.5
$R_{\rm free}$ (%)		20.9	23.4
r.m.s. ^b bonds (Å)		0.014	0.012
r.m.s. angles (°)		1.536	1.329

Table 3.1: Data collection and refinement statistics for $OatWY^{\perp}$.

^{*a*} Values in parentheses represent the highest resolution shell b r.m.s., root mean square

¹ The atomic coordinates and structure factors (codes 2WLC, 2WLD, 2WLE, 2WLF, and 2WLG) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

	OatWY+CoA	OatWY+AcCoA	OatWY+S-CoA ^c
Data collection	Culta	ALS 4 2 2	
Data collection	Cu Κα	ALS 4.2.2	CLS USID
Wavelength (Å)	1.5418	0.9790	0.9790
Resolution $(Å)^a$	40-2.20	40-2.35	40-1.90
	(2.28 - 2.20)	(2.48–2.35)	(1.97 - 1.90)
Space group	P222	P222	P222
a (Å)	79.07	78.30	78.67
b (Å)	94.52	94.52	94.39
c (Å)	100.89	100.59	100.87
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90
$R_{\rm sym}$ (%) ^a	5.7 (46.4)	9.6 (44.1)	5.1 (28.6)
$I/\sigma(I)^a$	34.5 (3.4)	15.0 (3.2)	52.8 (7.2)
Completeness $(\%)^a$	99.7 (97.4)	100 (100)	99.0 (95.2)
Unique reflections ^{<i>a</i>}	39,161 (3761)	32,150 (4618)	59,306 (5651)
Redundancy ^a	6.0 (5.6)	4.8 (4.9)	10.0 (8.9)
Refinement			
Average B factor $(Å^2)$			
Protein	21.9	12.5	15.1
Ligand	50.8	50.4	47.9
Water	31.2	24.9	27.5
Ramachandran statistics			
Favored regions (%)	98.3	97.3	97.6
Additionally allowed regions (%)	1.7	2.7	2.4
Disallowed regions (%)	0.0	0.0	0.0
R_{work} (%)	19.5	18.3	19.3
$R_{\text{free}}(\%)$	24.2	23.1	23.1
$r m s^{b} bonds (Å)$	0.008	0.009	0.011
r.m.s. angles (°)	1.104	1.468	1.342

Table 3.1: Data collection and refinement statistics for OatWY (continued).

^{*a*} Values in parentheses represent the highest resolution shell ^{*b*} r.m.s., root mean square ^{*c*} S-CoA denotes *S*-(2-oxopropyl)-CoA



Figure 3.2: Multiangle light scattering analysis of purified OatWY.

Each monomer of the OatWY homotrimer is composed of three domains. The C-terminal extension (residues 192–215) consists of a loop region followed by a short 3_{10} helix. The N-terminal domain (residues 6–191) is composed of a left-handed β -helical (L β H) motif containing seven turns of parallel β -helix. L β H domains are characterized by repeating left-handed connections of parallel β -strands. Each helical turn consists of three β -strands connected by short loops and displays a signature motif composed of the repeating hexapeptide sequence ((LIV)(GAED) X_2 (STAV)X). L β H folds are stabilized by both

intramolecular β-sheet hydrogen bonds and an inner hydrophobic core resulting from the packing of leucine and isoleucine residues at position 1 (typically termed "i") of each hexapeptide motif and have been observed in various acyltransferases (Raetz & Roderick, 1995, Beaman et al., 1997, Beaman et al., 1998, Wang et al., 2002, Lo Leggio et al., 2003, Kehoe et al., 2003). Our DNA sequencing results revealed that the OatWY construct has a random mutation at position 67 (N67I) relative to that reported in the EMBL⁺ database (accession number Y13969). This residue is located in position i of the interior core region of the LBH domain, which is characterized by an extremely hydrophobic environment. Interestingly, the observed Ile-67 mutation aligns and packs well within the Leu/Ile hydrophobic core typical of L^βH domains. In the OatWY homotrimer, a long extended loop region (residues 118–136) projects from the central motif of each LBH domain to interact with the C-terminal extension (residues 192'-215') of an adjacent monomer (Figure 3.3A and B). Through this intermolecular interface, each monomeric subunit is intimately associated with the other subunits of the homotrimer via a network of main chain and water-mediated hydrogen bonds and ionic interactions (a salt bridge between Arg-148 and Glu-92' of the adjacent monomer for example). An electrostatic surface calculation of the OatWY trimer shows a predominantly electronegative character in the region of the protruding $loop^{118-136}$ and the C-terminal extension, whereas the interior channel formed between the three L β H domains of the homotrimer is predominantly electropositive (Figure 3.3D and E).

^{*} EMBL Nucleotide Sequence Database, http://www.ebi.ac.uk/embl/



Figure 3.3: Quaternary structure and electrostatic surface map of OatWY.

(A) Structure of OatWY-acetyl-CoA complex viewed parallel to the 3-fold axis, and (B) structure viewed perpendicular to the 3-fold axis of the OatWY trimer (the three subunits are colored in blue, red, and green, with the N- and C-terminal ends indicated in the latter). The potential acceptor binding region is outlined by the black dotted box. Carbon atoms in the acetyl-CoA are represented as yellow, and non-carbon atoms are colored according to atom type (nitrogen, blue; oxygen, red; phosphorus, orange; and sulfur, gold). Two essential catalytic residues (His-121 and Trp-145') are shown as stick models and are indicated by arrows. (C) Potential acceptor binding region. Surfaceexposed residues potentially involved in polysaccharide acceptor binding are displayed in yellow for residues from one monomer and in cyan for residues from adjoining monomer. Bound CoA is shown as a stick model, and two conserved catalytic residues (His-121 and Trp-145) are represented as red sticks. Electrostatic surface representations (D, top view, and E, bottom view) along with the homotrimeric structure of OatWY show the overall electropositive charge (blue) at the monomer interface of the OatWY trimer. Arrows in the electrostatic surface maps indicate the location of substrate-binding sites between adjoining subunits. Electronegative surface charge is colored in red. (F) Movement of Tyr-171 upon donor binding. Superposition of the apo (yellow) and donorbound (blue) OatWY structures. Tyr-171 is shown in blue; carbon atoms in the acetyl-CoA molecule are represented as green, and non-carbon atoms are colored according to atom type (nitrogen, blue; oxygen, red; phosphorus, orange; and sulfur, gold).

3.3.2 Donor-binding Site

The co-substrate structures of OatWY in complex with the substrate acetyl-CoA and its analogues (CoA, S-(2-oxopropyl)-CoA) identify the donor-binding site as localized to the interface of adjacent monomers in the homotrimer. Specifically, the CoA moiety occupies a prominent cleft created collectively by the LBH domains localized to the Cterminal region of each monomer (Figure 3.3A and B). The donor forms polar and hydrophobic contacts with residues in both flanking monomers. Binding of donor appears to induce little overall conformational change in the enzyme as the apo and co-substrate structures can be overlapped with a root mean square deviation of 0.24–0.34 Å for all 630 $C\alpha$ atoms of the homotrimer. However, a few critical and we believe functionally important differences do arise upon donor binding, the most significant being the movement of the aromatic side chain of Tyr-171. This tyrosine is oriented such that the side chain hydroxyl points toward the LBH domain, thereby blocking the cleft created by interfacing subunits in the apo structure. In contrast, the aromatic side chain of Tyr-171 flips in the co-substrate structure, to contact directly the adenine ring of the coenzyme A via hydrophobic interactions (Figure 3.3F). In addition, the amine side chain of Lys-190' is ordered in the complex structure via a direct electrostatic interaction with the ribose 3'phosphate group of the CoA molecule. In general, the binding of CoA is stabilized by a prominent set of electrostatic interactions. In addition to Lys-190', the ribose 3'-phosphate group is also bound via a direct interaction with Lys-154. The ribose 2'-phosphate group forms water-mediated hydrogen bonds to Lys-136 and directly interacts with the side chain of Lys-154. The carbonyl oxygen of the phosphopantothenyl arm forms hydrogen bonds

to the main chain amide group of Ser-166'. The amide nitrogen located at the middle of the pantothenyl arm forms a direct hydrogen bond to Asp-119, and the amide nitrogen of the CoA hydrogen bonds with the carbonyl oxygen of Asp-119. The phosphopantothenyl arm of CoA is also stabilized by hydrophobic interactions with the side chains of apolar residues, including Val-163', Val-180', and Val-189' from one monomer and Ile-123 and Leu-153 from the adjoining monomer (Figure 3.4A). Importantly, the donor acetyl-CoA-bound structure (as compared with that with CoA) facilitates formation of an additional hydrogen bond between the acyl oxygen of acetyl-CoA and His-121, which we believe is one of the catalytic residues. Interestingly, our structure with the donor substrate analogue S-(2-oxopropyl)-CoA, an inhibitor of OatWY activity, binds in an identical fashion to that of acetyl-CoA except for the loss of this hydrogen bond to His-121, an interaction disfavored by the propyl functionality of the nonhydrolyzable S-(2-oxopropyl)-CoA.

Figure 3.4: Donor binding site of OatWY.

(A) Stereoview of the donor-binding site with electron density of acetyl-CoA in a refined $2F_o - F_c$ map contoured at 1.5 σ . Bound acetyl-CoA molecule is shown using green for carbon and phosphorus atoms, with other atoms colored according to atom type (nitrogen, blue; oxygen, red; and sulfur, gold). Interacting residues from one monomer are shown in yellow, and interacting residues from the adjoining monomer are shown in cyan. Water molecules are depicted as blue spheres, and His-121 and Trp-145' are displayed as red sticks. Polar contacts are shown as dotted lines. (B) Stereoview of a structural alignment between the complexes of OatWY with acetyl-CoA and a nonhydrolyzable CoA analogue. Key residues that interact with the donor substrate are represented in yellow for the acetyl-CoA complex and in blue for the *S*-(2-oxopropyl)-CoA complex. Acetyl-CoA and *S*-(2-oxopropyl)-CoA molecules are depicted in green and magenta, respectively. His-121 and Trp-145' are represented in red.



3.3.3 Acceptor Specificity of OatWY

A series of activity assays was carried out to examine the acceptor specificity of OatWY. All activity profiles were monitored by the spectrophotometric procedure described previously (Alpers et al., 1965) and in the presence of various acceptor candidates, including monomeric sialic acid (Neu5Ac), activated sialic acid (CMP-Neu5Ac), glucose, [Glc- $(\alpha 1 \rightarrow 4)$ -Sia] disaccharide, colominic acid (a homopolymer of sialic acid with $\alpha 2$,8-linkages), and the OatWY-specific polysaccharide ([$\rightarrow 6$)-Glc- $(\alpha 1 \rightarrow 4)$ -Sia- $(\alpha 2 \rightarrow)_n$ (Table 3.2). OatWY specifically catalyzed the *O*-acetyltransferase reaction in the presence of the *N. meningitidis* serogroup Y polysaccharide. Other substrates, including colominic acid, showed insignificant activities except for the disaccharide molecule, which acted as an acceptor with ~19% activity of the wild type enzyme. *S*-(2-oxopropyl)-CoA was also tested as a potential donor, along with the OatWY polysaccharide. The activity level of the reaction mixture containing the *S*-(2-oxopropyl)-CoA was dramatically reduced.

Acceptor ^a	Activity (%)
Polysaccharide $[Glc-(\alpha 1 \rightarrow 4)-Sia]_n$	100.0±0.3
Disaccharide [Glc-(α1→4)-Sia]	19.2±0.2
Colominic acid $[\alpha(2\rightarrow 8)Sia]_n$	2.1±0.2
Neu5Ac	0.3±0.2
CMP-Neu5Ac	0.3±0.1
Glucose	0.0±0.2
<i>S</i> -(2-oxopropyl)-CoA ^b	2.4±0.1
(-) control (water)	0.7±0.1

^a Concentrations of acceptors were adjusted to 5 mM sialic acid equivalent described in the experimental procedures.

^b Instead of acetyl-CoA, the S-(2-oxopropyl)-CoA was added to the assay mixture in the presence of the natural acceptor ([Glc-($\alpha 1 \rightarrow 4$)-Sia]_n).

3.3.4 Identification of Potential Catalytic Residues

To investigate the importance and roles of the highly conserved residues localized within the active site of OatWY (His-121 and Trp-145'), two mutant forms of the enzyme, H121A and W145A, were generated and analyzed for catalytic activity. In addition, a Y171A mutant was also generated to investigate potential effects on donor binding. The results showed that all three mutations dramatically reduced activity. The histidine mutant has only 2.0% of the activity of wild type protein. The tryptophan mutant showed 1.8% of the activity compared with wild type enzyme. Finally, the tyrosine mutant showed 2.1% of the activity of wild type OatWY (Table 3.3).

Enzyme	Activity (%)
Wild type	100.0±1.0
His121Ala	2.0±0.4
Trp145Ala	1.8±0.7
Tyr171Ala	2.1±0.7

Table 3.3: Comparison of activities of wild type and mutant OatWY.

3.3.5 Kinetic Analysis

A more complete kinetic analysis of OatWY was performed to further characterize the nature of the enzymatic reaction. All kinetic experiments were carried out at 25 °C, and data were fitted to the Michaelis-Menten equation. Kinetic parameters for wild type OatWY enzyme are summarized in Table 3.4. The K_m value for donor (acetyl-CoA) was 0.21 mM, and the K_m value for acceptor (Y polysaccharide) was 2.55 mg/ml, which is equivalent to 5.4 mM of the total [Glc-(α 1 \rightarrow 4)-Sia] disaccharide. Static light-scattering results showed that the estimated mass range of the polysaccharide is around 100–230 kDa, which is equivalent to 200–400 disaccharide units (data not shown). Based on this mass information, a K_m value of 11–26 μ M can be calculated for the polysaccharide. Specificity constants (k_{cat}/K_m) were 6.3 s⁻¹ mM⁻¹ for acetyl-CoA and 80–190 s⁻¹ mM⁻¹ for the polysaccharide.

Substrate	Acetyl CoA		Acceptor ^a			
	K_m	$k_{\rm cat}$	$k_{\rm cat}/K_m$	K_m	$k_{\rm cat}$	$k_{\rm cat}/K_m$
	μΜ	s^{-1}	$mM^{-1}s^{-1}$	μΜ	s ⁻¹	$mM^{-1}s^{-1}$
	210±24	1.3±0.1	6.3	11±1-26±3	2.1±0.1	80-190

Table 3.4: Kinetic parameters of OatWY.

^{*a*} Acceptor was the *N. meningitidis* serogroup Y polysialic acid, ([Glc-(α 1 \rightarrow 4)-Sia]_n).

3.4 Discussion

Capsular polysialic acids that decorate the cell surfaces of various bacteria act as important virulence factors in these pathogenic species that inflict serious human disease, including sepsis and meningitis (Vogel *et al.*, 1996, Vimr *et al.*, 2004). *O*-Acetylation, the only common modification of the bacterial sialic acid moiety, alters the physiological properties of the resulting modified bacterial polysaccharides (Orskov *et al.*, 1979, Szu *et al.*, 1991, Frasa et al., 1993, Fattom *et al.*, 1998). In this study, we provide the first structural characterization of a polysialic acid *O*-acetyltransferase, which is that of OatWY from *N. meningitidis*, providing a foundation for understanding previous biochemical analyses as well as the new kinetic specificity and mutagenesis data presented here.

The OatWY enzyme structure was solved as an intimately associated homotrimer held together by numerous noncovalent interactions, which localize primarily to the Cterminal extension (residues 192–215) and the inserted loop region (residues 118–136) from the β -helix coils of the L β H domain. As typical, the L β H domains of the trimer are stabilized by an extensive intermolecular hydrogen bonding network between β -strands and further stabilized by hydrophobic interactions formed by the regular arrangement of Leu and Ile packed within the interior core of the $L\beta H$ domain.

The L β H domains in OatWY show characteristic features of the repeating β -helix fold motif, each of which is composed of three β -strands (S1, S2, and S3) connected by short loop regions of 1-3 amino acids (L1, L2, and L3) (Iengar et al., 2006). The LBH fold is found in various bacterial acetyltransferases, including xenobiotic acetyltransferase from *Pseudomonas aeruginosa* (PaXAT) (Beaman et al., 1998), *N*-acetyltransferase from *C*. *jejuni* (PglD) (Rangarajan al., 2008). virginiamycin acetyltransferase D et from Enterococcus faecium (VatD) (Kehoe et al., 2003), and lac operon galactoside acetyltransferase (GAT) (Wang et al., 2002), UDP-N-acetylglucosamine acyltransferase (Raetz & Roderick, 1995), and maltose acetyltransferase (MAT) (Lo Leggio et al., 2003) from E. coli. These proteins are usually composed of an α -helical or α/β domain in addition to the LBH domain, the former playing roles in oligomerization and "capping" of the LBH domain. Interestingly, OatWY appears to be a minimalized form of these enzymes, with the L β H domain being the only major structural domain and the small Cterminal extension composed only of a loop terminated by a short 3_{10} helix.

A tandemly repeated hexapeptide sequence typically defines the L β H fold in bacterial acetyltransferases (Beaman et al., 1998, Wang et al., 2002, Lo Leggio et al., 2003). The aliphatic residues (Leu, Ile, or Val) at position *i* of the hexapeptide motif ((LIV)(GAED) X_2 (STAV)X) project into the interior of the β -helical domain. Interestingly, OatWY contains an unusual set of hexapeptide-repeating motifs, with atypical residues at position *i* + 1 or *i* + 4 except for those hexapeptides initiating within sequence motifs 61 (IADDVE), 140 (IGNHVW), 146 (LGRNVT), and 164 (VGSHTV) (Figure 3.5A). The

first five coils of OatWY are also longer and more sequence variable than the more regularly repeating hexapeptides found in other bacterial acetyltransferases (Figure 3.5). The irregular hexapeptide motifs of OatWY result in a larger size for the first five β -strand coils and lead in turn to a less compact overall L β H structure. In addition, the size of the L β H domain in OatWY (7 β -helical coils) is significantly extended compared with other bacterial acetyltransferase structures, which typically utilize ~5 β -helical coils in the formation of the L β H domain.

The potential functional implications of these alterations in sequence and number of repeating patterns of the L β H motif can be illustrated by comparison of the geometric features and overall span of the trimeric L β H domain in OatWY and other bacterial acetyltransferases. In OatWY, the axis of the L β H domain makes an angle of 34° with the 3-fold axis of the trimer, strikingly larger than any angle created by other bacterial acetyltransferases containing the β -helical fold and in general very unusual for the family of hexapeptide repeat-containing proteins. Other bacterial sugar acetyltransferases reported to date typically show a nearly parallel arrangement of β -helical domain axes relative to the trimeric axis. For example, in the UDP-*N*-acetylglucosamine acyltransferase LpxA there is a 1–2° angle between axes (Williams *et al.*, 2006), whereas the angle in the *lac* operon GAT or MAT is 10–12° (Wang et al., 2002, Lo Leggio et al., 2003). In addition to the more splayed nature of the OatWY homotrimer, the greater number of L β H repeats in OatWY results in a longer span of the L β H domain (~34 Å compared with the 18–20 Å range in other bacterial acetyltransferases) (Figure 3.5).

Figure 3.5: Structural comparison of OatWY with other bacterial acetyltransferases containing L\$H motifs.

(A) polysialic acid *O*-acetyltransferase OatWY; (B) xenobiotic acetyltransferase PaXAT (PDB access code 2XAT); (C) GAT (PDB access code 1KRU); (D) MAT (PDB access code 1OCX). Only two monomeric subunits of the trimer are represented for clarity (in green and red). Angles between the 3-fold axis and L β H domains are illustrated with two-headed arrows along with the measured overall span of the L β H domains. Below the OatWY structure in (A) is the signature sequence of the hexapeptide-repeating motif (LIV)(GAED)X₂(STAV)X displayed with positional indicators (*i*, *i* + 1, *i* + 2...) as well as the hexapeptide sequence motifs that define the OatWY L β H domain. Sheets and loops composed of the L β H domain are represented as S1–3 and L1–3, respectively. Sheets (S1–S3) are boxed with a yellow color, and the hexapeptide sequences that obey the rule ((LIV)(GAED)X₂(STAV)X) are boxed with solid black rectangles, and those that do not with dashed red rectangles. The defining hexapeptide repeating motifs of each of the other acetyltransferases are also represented in a sequence table below.





It seems likely that these differences in span and dramatic inclination of the angle in the OatWY trimer relate to its substrate specificity, because it binds to extended polymers of polysialic acids, which are much larger than the monosaccharide acceptor sugar substrates of other characterized sugar acetyltransferase families. Supporting this hypothesis, the polysialic acid *O*-acetyltransferase NeuO from *E. coli* K1, a close homologue of OatWY, was recently shown to display optimal activity against a polysialic acid longer than 14 sialic acid residues (Bergfeld et al., 2007).

Our co-substrate structures show that the acetyl coenzyme A and its analogues define the OatWY active site as created at the interface of adjacent monomeric subunits, supporting the requirement of an active homotrimeric form for the enzyme. The donor substrate binds within a deep cleft that is characterized by the presence of two highly conserved residues, His-121 and Trp-145'. The CoA is held by several noncovalent interactions to the active site, including a striking set of hydrophobic contacts between the adenine ring and the aromatic side chain of Tyr-171. Comparison with the apo structure shows that Tyr-171 likely plays a key and unique structural role in promoting enzyme activity, because donor binding induces a significant movement of the Tyr-171 side chain from a position where it blocks entry of the catalytic cleft (apo-form) to one that allows the observed ring stacking with the adenine ring of the coenzyme A moiety (donor-bound form; Figure 3.3F). The potential importance of the observed tyrosine-mediated donor binding for OatWY function is further confirmed by our mutagenesis studies, which showed that a Y171A mutant form of the enzyme had dramatically reduced activity. The active site of OatWY is also uniquely characterized by a number of positively charged residues including Arg-116, Arg-148', and His-167', which can potentially bind to and position the negatively charged terminal sugar of the incoming polysaccharide acceptor molecule for acetylation. Surface-exposed residues in the potential acceptor binding region (Figure 3.3C) also provide a positively charged surface patch, including that arising from the side chains of Arg-51, Lys-53, His-75, and Lys-98 from one interacting subunit, and Lys-44', Lys-85', His-111', Arg-197', and Lys-198' from the adjacent subunit. This capacious acceptor site, created by the large angle subtended by the monomer of OatWY with the trimeric axis (as described above), presents an appropriately sized and charged surface to interact with its negatively charged poly-glucosylsialic acid acceptor substrate.

The structure of OatWY in complex with substrate acetyl-CoA shares many of the features identified in the CoA-bound structure, interactions that ideally position the acetyl group in close proximity to the conserved His-121 and Trp-145', in a manner compatible with a role in mediating the transfer reaction. Sequence alignments of OatWY with other bacterial acetyltransferases, including MAT, GAT, VatD, PaXAT, and NeuO, clearly show that these residues are well conserved (Figure 3.6).

Figure 3.6: Multiple sequence alignment of OatWY with other bacterial *O*-acetyltransferases.

Conserved catalytic residues (His-121 and Trp-145') are highlighted in gray. The numbering of the *N. meningitidis* sequence is shown. Residues involved in the protruding loop region are italicized, underlined, and highlighted in gray. Sequence alignments were calculated using the program ClustalW⁺ (Thompson *et al.*, 1994). Sequences from the following bacterial species are shown (TrEMBL[#] accession code: OatWY–*N. meningitidis* serogroup Y (Q93S40), NeuO–*E. coli* K1 (Q58WP5), VatD–*E. faecium* (P50870), MAT–*E. coli* K1 (P77791), PaXAT–*P. aeruginosa* (Q02HJ8), GAT–*E. coli* K1 (P07464)).

[•] ClustalW2, http://www.ebi.ac.uk/Tools/clustalw2/index.html

[#] TrEMBL: Computer-annotated supplement to Swiss-Prot, http://www.expasy.ch/sprot/

OatWY-N.mening	MGTHMYSEQGINNTINISTTSLTNATQLTVIGNNNSVYIGNNCKIVSSNI	50
MAT-E.coli	STEKEKMIAGELYRSADETLSRDRLRARQLIHRYNHSLAEE	41
GAT-E.coli	ERITAGKLFTDMCEGLPEKRLRGKTLMYEFNHSHPSE	43
VatD-E.faecium	MGPNPMKMYPIEGNKSVQFIKPILEKLENVEVGEYSYYD-SKNG	43
PaXAT-P.aerug	NPNIRVGRYSYYSGYYHG	37
NeuO-E.coli K1	MLRLKTQDSRLKTQDSRLKTQDSRLKTQDSRLKTQDSRLKTQDSRLKTQDSRLKTQDSRL	60
OatWY-N.mening	RLKGNNITLFIADDVENMGLVCSLHSDC	78
MAT-E.coli	HTLRQQILADLFGQVTEAYIEPTFRCDYGYNI	73
GAT-E.coli	VEKRESLIKEMFATVGENAWVEPPVYFSYGSNI	76
VatD-E.faecium	ETFDKQILYHYPILNDKL	61
PaXAT-P.aerug	HSFDDCARYLMPDRDDVDKL	57
NeuO-E.coli K1	KTQDSRLKTQDSFSVDDNGSGNVFVCGDLVNSKENKVQFNGNNNKLIIEDDVECRWLTVI	120
OatWY-N.mening	SLQIQAKTTMGNGEITIAEKGKISIGKDCMLAHG	112
MAT-E.coli	FLGNNFFANFDCVMLDVCPIRIGDNCMLAPG	104
GAT-E.coli	HIGRNFYANFNCIIVDDYTVTIGDNVLIAPN	107
VatD-E.faecium	KIGKFCSIGPGVTIIMNG-ANHRMDGSTY	89
PaXAT-P.aerug	VIGSFCSIGSGAAFIMAGNQGHRAEWAST	86
NeuO-E.coli K1	FRGDNNYVRIHKNSKIKGDIVATKGSKVIIGRRTTIGAGFEVVTDKCNVTIGHDCMIARD	180
	121 145 171	
OatWY-N.mening	YEIR <u>NTDMHPIYSLENGERINHGK</u> DVIIGNHVWLGRNVTILKGVCIPNNVVVGSHTVLYK	172
MAT-E.coli	VHIYTAT-HPIDPVARNSGAELGKPVTIGNNVWIGGRAVINPGVTIGDNVVVASGAVVTK	163
GAT-E.coli	VTLSVTG-HPVHHELRKNGEMYSFPITIGNNVWIGSHVVINPGVTIGDNSVIGAGSIVTK	166
VatD-E.faecium	PFNLFGN-GWEKHMPKLDQLPIKGDTIIGNDVWIGKDVVIMPGVKIGDGAIVAANSVVVK	148
PaXAT-P.aerug	FPFHFMH-EEPAFAGAVNGYQPAGDTLIGHEVWIGTEAMFMPGVRVGHGAIIGSRALVTG	145
NeuO-E.coli K1	VILRASDGHPIFDIHSKKRINWAKDIIISSYVWVGRNVSIMKGVSVGSGSVIGYGSIVTK	240
OatWY-N.mening	SFKEPNCVIAGSPAKIVKENIVWGRKMYHSTMYDDPTLNEFYK	215
MAT-E.coli	DVP-DNVVVGGNPARIIKKL	182
GAT-E.coli	DIP-PNVVAAGVPCRVIREINDRDKHYYFKDYKVESSV	203
VatD-E.faecium	DIA-PYMLAGGNPANEIKQRFDQDTINQLLDIKWWNWPIDIINENIDKILDNSIIREVIW	207
PaXAT-P.aerug	${\tt DVE-PYAIVGGNPARTIRKRFSDGDIQNLLEMAWWDWPLADIEAAMPLLCTGDIPALYQH}$	204
NeuO-E.coli K1	DVP-SMCAAAGNPAKIIKRNIIWARTDKAELISDDKRCSSYHAKLTQ	286
OatWY-N.mening		
MAT-E.coli		
GAT-E.coli		
VatD-E faecium	KK 209	

VatD-*E.faecium* KK----- 209 PaXAT-P.aerug WK<u>O</u>RQATA 212 NeuO-*E.coli K1* ----- Based on previous studies of bacterial acetyltransferases (Wang et al., 2002) and our current structural, mutagenesis, and kinetic information, a mechanism for acetyl transfer reaction to the poly-glucosylsialic acid substrate can now be proposed (Figure 3.7). Trp-145' is well positioned to interact in a typical hydrophobic stacking interaction with the six-membered ring of the sialic acid, thereby orienting the polysaccharide properly for acetyl transfer. Upon donor binding, Tyr-171 alters its original conformation to mediate hydrophobic interactions with the adenine group of the acetyl-CoA. The catalytic residue, His-121 from the adjoining subunit, abstracts the proton from the hydroxyl group (O-7 or O-9) of the polysialic acid with assistance from the main chain carbonyl oxygen of Arg-197' in the adjacent subunit. This promotes the nucleophilic attack of the hydroxyl group on the carbonyl carbon of the acetyl-CoA, hence transfer of the acetyl group to the sialic acid.



Figure 3.7: Mechanistic scheme of OatWY polysialic acid acetyltransferase.

In the schematic above, the two interacting subunits, which define a complete active site, are represented as gray boxes and serve to illustrate the conformational role of Tyr-171 in allowing access to substrate acetyl-CoA. In the dotted box below, a proposed mechanistic scheme is shown highlighting the role of His-121 from one subunit and Trp-145' from the second subunit that make up the active site.

Our complex structure with the nonhydrolyzable acetyl-CoA analogue, S-(2-oxopropyl)-CoA, further supports this mechanistic proposal (Figure 3.4B). The primary structural and functional difference between the S-(2-oxopropyl)-CoA complex and that of the substrate acetyl-CoA is the presence of a methylene group between the sulfhydryl group of the CoA and the carbonyl carbon of the acetyl moiety, a chemical feature that renders the substrate inert, thereby inhibiting the enzymatic reaction competitively. Our structure shows this methylene group of the nonhydrolyzable S-(2-oxopropyl)-CoA is stabilized through additional hydrophobic contacts with Met-108 and Ala-110 in the enzyme active site.

Our generated activity profiles with various sugar compounds, including Neu5Ac, CMP-Neu5Ac, glucose, [Glc- $(\alpha 1 \rightarrow 4)$ -Sia] disaccharide, colominic acid, and Y polysaccharide ([\rightarrow 6)-Glc- $(\alpha 1 \rightarrow 4)$ -Sia- $(\alpha 2 \rightarrow]_n$), indicate that OatWY specifically reacts with Y meningococcal polysaccharides. These results clearly show that OatWY is highly specific for the unique form of the heteropolymeric polysialic acid described above and does not bind to the homopolymeric polysaccharide of *E. coli* K1 or *N. meningitidis* serogroup B (colominic acid, [α (2 \rightarrow 8)Sia]_n). Other substrate candidates also did not show detectable activities. The exquisite acceptor specificity of OatWY was further investigated by kinetic analysis and yielded a K_m value for the polysaccharide of 2.55 mg/ml (\sim 11–26 μ M, when calculated on the basis of the average molecular mass of 100–230 kDa estimated from the light-scattering analysis). Because the sequence of OatWY from W-135 meningococci was reported to be 100% identical to the one from Y meningococci (Claus et al., 2004), we can predict that the W polysaccharide ([\rightarrow 6)-Gal-(α 1 \rightarrow 4)-Sia-(α 2 \rightarrow]_n) would also be an acceptor for OatWY.

Molecular modeling of a truncated piece of the polysaccharide acceptor model from Y meningococci, specifically $[\rightarrow 6)$ -Glc- $(\alpha 1 \rightarrow 4)$ -Sia- $(\alpha 2 \rightarrow)_4$, with the crystal structure of OatWY showed that the acceptor substrate can be readily accommodated, in terms of size and potential electrostatic interactions within the proposed acceptor binding region. The surface-exposed residues described above, His-75 and Lys-98 from one subunit and His-111', Arg-148', and Arg-197' from the adjoining subunit, provide many direct polar and electrostatic contacts to the negatively charged C-2 carboxylate and hydroxyl groups of the modeled acceptor polysialic acid. In addition to the major interactions with the positively charged residues, negatively charged and polar residues such as Ser-73 and Glu-92 are also poised appropriately for acceptor binding in our model. Other positively charged surface residues "downstream" from our modeled acceptor (localized in the N-terminal region of the LBH domain, including Arg-51 and Lys-44') may potentially provide additional electrostatic interactions in the longer, physiological polyglucosylsialic acceptors. Future direct support illustrating acceptor binding will hopefully be achieved once the production of homogeneous mixtures of suitably sized sugar polymers becomes feasible.

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<u>CHAPTER 4</u>: STRUCTURAL BASIS FOR ACCEPTOR BINDING OF THE SIALYLTRANSFERASE CST-II FROM *CAMPYLOBACTER JEJUNI*[#]

4.1 Introduction

The human pathogen *Campylobacter jejuni* is recognized as the leading causative agent of bacterial diarrhea and food borne gastroenteritis worldwide (Nachamkin *et al.*, 1998, Mead *et al.*, 1999). This organism has been shown to express variable outer core structures of lipooligosaccharide (LOS), which mimic the carbohydrate moieties of human gangliosides. Because of the molecular mimicry, the LOS of *C. jejuni* is considered a major virulence factor providing a protective barrier to evade host's immune system (Gilbert *et al.*, 2002, Guerry *et al.*, 2002). In addition, the carbohydrate mimicry between LOS outer core structures of *C. jejuni* and human gangliosides has been suggested to trigger the development of autoimmune diseases such as Guillain–Barré syndrome (Endtz *et al.*, 2000, Yuki *et al.*, 2004). Oligosaccharides found in the outer core region of LOS of various *C. jejuni* strains contain terminal sialic acid residues, which provide the diversity in these structures (Gilbert et al., 2002).

Sialic acids (*N*-acetylneuraminic acid, or NeuNAc) are a family of nine carbon keto sugars, which participate in various biologically important processes including cellular recognition, microbial infection, and tumor metastasis (Angata & Varki, 2002, Hakomori, 2002, Schauer, 2004, Olofsson & Bergstrom, 2005, Saez-Llorens & McCracken, 2003).

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They are widely disseminated molecules not only in mammalian tissues but as well in pathogenic bacteria (Severi *et al.*, 2007). In mammals, sialic acids typically exist as terminal residues on the outermost cell surface glycoconjugates (Varki, 1997, Traving & Schauer, 1998). For example, gangliosides located predominantly in vertebrate nerve cell surfaces are complex glycosphingolipids composed of oligosaccharide chains containing one or more terminal sialic acids (Vyas & Schnaar, 2001). Sialic acids also have been found as terminal residues on the surface of a number of pathogenic bacteria and shown to play important roles during infection (Severi et al., 2007, Preston *et al.*, 1996, Saez-Llorens & McCracken, 2003). A recent study of the human pathogen *C. jejuni* revealed that a sialylated variant of this bacteria showed significantly higher invasion level for epithelial cells compared to unsialylated one (Louwen *et al.*, 2008).

Sialyltransferases are enzymes that transfer sialic acids from an activated sugar donor (CMP-Neu5Ac) to various acceptor molecules including *N*-acetylgalactosamine (GalNAc), galactose, or another sialic acid located at terminal positions of oligosaccharides of glycoproteins and glycolipids with distinct linkages based on their specificities (Cantarel *et al.*, 2009, Harduin-Lepers *et al.*, 1995). They are classified into several glycosyltransferase (GT) families according to sequence similarities in the CAZy database (<u>Carbohydrate-Active enZyme, http://www.cazy.org</u>). Bacterial sialyltransferases are mainly categorized into three distinct families (GT-42, GT-52, and GT-80) except polysialyltransferases in GT-38.

Cst-II (GT42) from the strain OH4384 of *Campylobacter jejuni* is a bifunctional sialyltransferase, transferring sialic acid to the terminal galactose moiety of the

lipooligosaccharide with an α -2,3-linkage, and subsequently transferring another sialic acid to the initially formed sialoside with an α -2,8-linkage (Gilbert *et al.*, 2000). The structure of Cst-II in complex with the inert donor analogue, CMP-3FNeu5Ac showed a significant variation of the GT-A fold and the absence of a DXD motif which contributes directly to catalysis in other glycosyltransferase families (Chiu *et al.*, 2004). Structural and kinetic analysis of Cst-II supported a catalytic mechanism involving a conserved histidine residue as general base (His-188) which deprotonates the acceptor followed by direct attack of the acceptor on the anomeric carbon (C-2) of the donor substrate (CMP-Neu5Ac) in the sialic acid transfer event. While the previously reported structure of Cst-II provided valuable information for donor binding, the lack of structural information on the acceptor sugar binding mode has hampered our ability to understand the molecular basis for acceptor binding specificity and catalytic mechanism of this important class of glycosyltransferase.

Here, we report the structure of Cst-II in complex with the fragment of the donor substrate, CMP and the terminal trisaccharide of its natural acceptor, Neu5Ac- α -2,3-Gal- β -1,3-GalNAc. In addition, essential residues which we observe to directly bind to the sialic acid moiety of the acceptor were examined structurally and kinetically. This work provides the first structural basis of acceptor binding during the LOS sialylation of *C. jejuni* and significantly improves our understanding of the substrate specificity and mechanistic features of bacterial sialyltransferases.

4.2 Experimental Procedures

4.2.1 Cloning, Overexpression, and Purification of Cst-IIA32 and its Mutants

All molecular biology procedures were carried out similarly described in the previous paper (Chiu et al., 2004). Briefly, the truncated version of Cst-II (32 residues from C-terminus) were cloned in pET-28a vector (Novagen) with the N-terminal hexahistidine tags and subsequently transformed into electrocompetent *E. coli* cells (BL21 λ DE3, Novagen) for expression. To produce active site mutants, site-directed mutageneses were performed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions with following primers (Integrated DNA Technologies). Cst-II N51A-F (5'-

GGTAAAAAATGCAAGGCAGTATTTTACGCTCCTATTCTTTTTTGAAC-3') and Cst-II N51A-R (5'-

GTTCAAAAAAAAGAATAGGAGCGTAAAATACTGCCTTGCATTTTTACC-3') were Y81F-F N51A (5'used to generate the mutant. Cst-II CCGAACTAATTATGTGTTCTAATTTCAACCAAGCTCATCTAG-3') and Cst-II Y81F-R (5'-CTAGATGAGCTTGGTTGAAATTAGAACACATAATTAGTTCGG-3') were used to generate the Y81F mutant. Cst-II N51A/Y81F double mutant was constructed by using Cst-II N51A clone as a template with the forward primer Cst-II Y81F-F and the reverse primer Cst-II Y81F-R. All mutations were confirmed by DNA sequencing (Genewiz) and colonies with desire mutations were transformed into competent cells described above. Expression and purification of wild type and mutant enzymes were performed as described previously (Chiu et al., 2004).

4.2.2 Crystallization and Data Collection

Crystals of wild type Cst-IIA32 and its mutants were grown in similar conditions described previously (Chiu et al., 2004). Briefly, 8 mg/ml of purified protein mixtures containing 10 mM CMP, MgCl₂, and disaccharide (Gal-β-1,3-GalNAc) or trisaccharide (Neu5Ac-α-2,3-Gal-β-1,3-GalNAc) acceptor were crystallized in the mother liquor containing 100 mM HEPES, pH 7.5, 8% (w/v) polyethylene glycol 6000, and 5% (v/v) 2methyl-2,4-pentanediol by the hanging-drop vapor diffusion technique at 21 °C. The ternary complex of wild type Cst-II Δ 32 belonged to the space group P4 with unit cell dimensions a = 117.67, b = 117.67, c = 46.60 Å, and contained two molecules in the asymmetric unit. The crystal of Y81F mutant belonged to the same space group as wild type enzyme (P4) with similar unit cell dimensions (a = 116.62, b = 116.62, c = 46.84 Å) and also contained two molecules in the asymmetric unit. The crystal of N51A mutant belonged to the space group I4 and contained one molecule in the asymmetric unit with unit cell dimensions a = 116.19, b = 116.19, c = 46.95 Å. The crystals were cryoprotected in mother liquor containing 15% MPD and directly plunged into liquid nitrogen prior to data collection. X-ray diffraction data were collected at 100 K under a nitrogen stream using an in-house CuKa rotating anode x-ray generator coupled to a Mar345 detector or using a synchrotron source (beamline 08ID-1 of the Canadian Light Source (Saskatoon, SK) coupled to a Mar300CCD detector). Collected data were processed by the HKL2000 or MOSFLM suites (Leslie, 1992, Øtwinowski Z., 1997).

4.2.3 Structure Determination and Refinement

All structures were solved by molecular replacement with PHASER (McCoy, 2007) using the monomer of wild type Cst-II Δ 32 (PDB accession code: 1RO8) as the starting model. Subsequent manual model building was performed by COOT (Emsley & Cowtan, 2004) and refinement were carried out by REFMAC5 (Murshudov *et al.*, 1997) excluding 5% of reflections for the *R*_{free} calculation. In addition, Translation/Libration/Screw motion determination was incorporated into the procedure for further rounds of crystallographic refinement (Painter & Merritt, 2006). The trisaccharide ligand files were generated from the Dundee PRODRG server (Schuttelkopf & van Aalten, 2004). All structural figures were generated using PyMOL (DeLano, 2002) and electrostatic surface calculations were performed with the APBS plugin (Baker *et al.*, 2001). Final models were validated with MOLPROBITY (Davis *et al.*, 2007).

4.2.4 Synthesis of Acceptor Substrates

Synthetic di- and tri-saccharides used in this study (Gal- β -1,3-GalNAc- α -OBn, Neu5Ac- α -2,3-Gal- β -1,3-GalNAc- α -OBn, and Neu5Ac- α -2,3-Gal- β -1,3-GalNAc- β -pNP) were prepared in parallel from the corresponding monosaccharides (GalNAc- α -OBn and GalNAc- β -pNP) by glycosyltransferases-catalyzed addition of galactose and sialic acid as described below. GalNAc- α -OBn or GalNAc- β -pNP were dissolved at final concentrations in the reaction of 15 mM and 4 mM, respectively, in 50 mM NaOAc, pH 6.0, containing 10 mM MnCl₂, 1 mM DTT, UDP-galactopyranose (1.1 eq.), alkaline phosphatase (Sigma, 10–15 units/mL) and recombinant 1,3-galactosyltransferases

 $(CgtB_{OH4384}\Delta C30$ -C-term MalE (Bernatchez *et al.*, 2007), 0.1 mg/ml in reaction). The mixtures were monitored by TLC (mobile phase 7:2:1 ethyl acetate/methanol/water) until starting material was consumed. The reaction mixture was diluted with an equal volume of cold methanol before centrifugation (2 min, $10,000 \times g$) to remove solids. The supernatant was lyophilized and applied to a tC18 SepPak cartridge (Waters). After washing the cartridge with water and 5% methanol, products were eluted with higher concentrations of methanol (10-20 %), concentrated, re-dissolved in water, and freeze dried. To obtain pure disaccharides, the tC18 SepPak purification was repeated several times. То obtain trisaccharide, the partially purified material was dissolved in 50 mM HEPES; pH 7.5, containing 10 mM MnCl₂, 10 mM MgCl₂, 4mM DTT, CMP-Neu5Ac (1.0 eq. based on monosaccharide starting material) and alkaline phosphatase (Sigma, 10-15 units/mL) at an estimated acceptor concentration of 2.5 mg/ml. Reaction was initiated by addition of sialyltransferase (Cst-I (Chiu et al., 2007), <0.1 mg/ml in reaction mixture). After standing overnight, the reaction mixture was applied to a tC18 SepPak cartridge. The cartridge was then washed with water and the impure trisaccharide was eluted with 10% methanol. Fractions containing the target were pooled and concentrated before further purification in water on a column of Biogel P-2. Combined yields for the two step process were 30-50%.

Data for 4-Nitrophenyl (5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2ulopyranosylonic acid)-(2 \rightarrow 3)- β -D-galactopyranosyl-(1 \rightarrow 3)-2-acetamido-2-deoxy- β -Dgalactopyranoside. ¹H NMR (400MHz, D₂O, δ) 8.17 (d, 2H, Ar, J = 9.4 Hz), 7.12 (d, 2H, Ar, J = 9.4 Hz), 5.23 (d, 1H, H-1, $J_{1,2} = 8.5$ Hz), 4.48 (d, 1H, H-1', $J_{1',2'} = 7.8$ Hz), 4.27 (dd, 1H, H-2, $J_{2,3} = 10.8$ Hz), 4.20 (d, 1H, H-4, $J_{4,3} = 3.0$ Hz), 4.00 (dd, 1H, H-3', $J_{3',2'} = 9.8$ Hz, $J_{3',4'} = 3.1$ Hz), 3.94 (dd, 1H, H-3), 3.88-3.46 (m, 15H), 2.67 (dd, 1H, H- 3_{eq} , J_{3eq}

4.2.5 Enzyme Kinetics

Michaelis-Menten kinetic parameters were determined for Cst-II and mutants through variation of the concentration of acceptor at a fixed concentration of CMP-Neu5Ac. Enzyme concentrations were calculated using the method of Bradford (Bradford, 1976). Reaction rates were determined using a continuous coupled spectrophotometric assay, as previously described (Gosselin *et al.*, 1994, Chiu et al., 2004). Briefly, the release of CMP was coupled to the oxidation of NADH ($\lambda = 340$ nm, $\varepsilon = 6.22$ mM⁻¹cm⁻¹) through the action of nucleoside monophosphate kinase (NMPK), pyruvate kinase (PK), and lactate dehydrogenase (LDH). Both the enzymatic and the spontaneous hydrolysis of CMP-Neu5Ac were measured and were subtracted from rate measurements. Assays were conducted at 37 °C in a 200 µl volume containing 20 mM HEPES; pH 7.5, 50 mM KCl, 1% (w/v) BSA, 10 mM MnCl₂, 10 mM MgCl₂, 0.7 mM phosphoenolpyruvate , 0.35 mM NADH, 2 mM ATP, 5.9U of PK, 8.4U of LDH, and 7.9U of NMPK, 0.75 mM CMP-

Neu5Ac, and varying concentration of acceptor. The assay was initiated after the rate of NADH oxidation had stabilized by addition of enzyme with thorough mixing. Initial rates were plotted against acceptor substrate concentration, and K_m and V_{max} were calculated from the best fit of the data to the Michaelis-Menten equation using non-linear regression analysis with GraFit 5.0 (Erithacus Software).

4.3 Results

4.3.1 Overall Structure

Structures of the ternary complex of wild type Cst-II Δ 32 and various mutants in complex with the donor analogue CMP were solved via molecular replacement using the previously determined structure of native Cst-II (Chiu et al., 2004) as a search model. The high resolution edge of the resulting diffraction data varied from 1.95 to 2.20 Å, and the resulting structures are well refined, with R_{work}/R_{free} values ranging from 17/20% to 18/23%. Full details of the data collection and refinement statistics are summarized in Table 4.1. The crystal structure of the newly solved Cst-II Δ 32 in complex with the donor analogue (CMP) and the trisaccharide acceptor (Neu5Ac- α -2,3-Gal- β -1,3-GalNAc) maintains a highly similar overall architecture to the previously reported Cst-II structure bound to the nonhydrolyzable donor analogue (CMP-3FNeu5Ac) (Chiu et al., 2004), with a root mean square deviation of 0.37–0.43 Å on 241 C- α atoms. This earlier structure was shown to form an intimately associated homotetramer (space group P2₁), with all 4 expected membrane associating C-terminal domains aligned on the same face of the tetramer, a likely physiological requirement supported by static light scattering analysis in solution.

	Cst-II + CMP	N51A + CMP	Y81F + CMP
	+Trisaccharide		
Data collection	CLS 08ID	Cu Ka	Cu Ka
Wavelength (Å)	0.9793	1.5418	1.5418
Resolution $(Å)^a$	40-1.95	35-2.00	35-2.20
	(2.02 - 1.95)	(2.11 - 2.00)	(2.28 - 2.20)
Space group	<i>P</i> 4	<i>I</i> 4	<i>P</i> 4
<i>a</i> (Å)	117.67	116.19	116.62
<i>b</i> (Å)	117.67	116.19	116.62
<i>c</i> (Å)	46.60	46.95	46.84
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90
$R_{\rm sym}$ (%)	8.1 (36.8)	8.4 (40.8)	4.6 (25.3)
$I/\sigma(I)^a$	38.3 (5.0)	16.6 (3.3)	17.7 (2.7)
Completeness (%)	99.6 (96.9)	100 (100)	94.2 (87.7)
Unique reflections	46,782 (4,468)	21,401 (3,099)	30,500 (2,798)
Redundancy	6.3 (5.6)	4.4 (4.3)	2.3 (1.8)
Refinement			
$R_{ m work}$ (%)	16.4	18.6	17.7
$R_{\text{free}} (\%)$	19.8	23.0	22.8
r.m.s. ^{b} bonds (Å)	0.008	0.007	0.011
r.m.s. angles (°)	1.068	0.986	1.228
Average B-factor ($Å^2$)			
Protein	22.4	13.3	23.3
Ligand	42.8	22.8	40.3
Water	32.9	23.7	28.7
Ramachandran plot			
Most favored (%)	98.7	98.8	97.6
Allowed (%)	1.3	1.1	2.4

Table 4.1: Data collection and refinement statistics^{*}.

^{*a*} Values in parentheses represent the highest resolution shell ^{*b*} r.m.s., root mean square

^{*} The atomic coordinates and structure factors (codes 2X61, 2X62, and 2X63) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

Our structures of the Cst-II Δ 32 ternary complex and Y81F mutant presented in this work belong to the space group P4, containing two molecules in the asymmetric unit, and the N51A mutant belongs to the space group I4, containing one molecule in the asymmetric unit, such that identically arranged tetrameric structures as observed in the earlier monoclinic space group can be generated around the four-fold crystallographic axis of these tetragonal space groups.

4.3.2 Donor-binding Site

The ternary complex structure of Cst-II Δ 32 shows clear electron density for both CMP in the donor site and the trisaccharide acceptor. The amino acids that line the donor binding site of the complex adopt a highly similar conformation as to that observed in the previously reported Cst-II structure in complex with the physiological sugar donor CMP-3FNeu5Ac. All key intermolecular contacts are maintained including the stabilization of the phosphate moiety of CMP by the conserved Asn-31, Tyr-156, and Tyr-162 residues. Interestingly, the flexible lid domain (residues 155–188) which was observed to fold over the active site upon donor binding in the previous Cst-II - CMP-3FNeu5Ac donor complex is partially disordered in structures of the wild type ternary complex (Cst-II, CMP, Neu5Ac- α -2,3-Gal- β -1,3-GalNAc acceptor) and Y81F (CMP) mutant presented here, suggesting a possible role for the donor sugar Neu5Ac in ordering the lid. However, the lid domain adopts a highly ordered, completely closed form in the new structure of the N51A (CMP) mutant, highly similar to that of the earlier Cst-II - CMP-3FNeu5Ac donor complex.

4.3.3 Acceptor-binding Site

The physiological trisaccharide acceptor (Neu5Ac- α -2,3-Gal- β -1,3-GalNAc) binds to the open cleft of Cst-II at the C-terminal half of the Rossmann domain adjacent to the donor-binding site (Figure 4.1A). The acceptor substrate binds to the enzyme through a number of hydrogen bonds to the *N*-acetylamino group of the terminal sialic acid moiety. The N-acetylamino group interacts with the side chains of both the conserved Asn-51 and Tvr-81. The guanidinium side chain of Arg-129 establishes hydrogen bonds to the hydroxyl group at C-7 of the sialic acid moiety and the hydroxyl group at C-2 of the galactose moiety of the trisaccharide acceptor. In addition, the carboxyl group of the acceptor sialic acid interacts with Ser-53, and the C-4 hydroxyl group with the carbonyl of Ser-79 (Figure 4.1C). There are also water-mediated hydrogen bonds between the sialic acid moiety of the trisaccharide and residues lining the acceptor-binding site. For example, the hydroxyl groups at C-7 and C-4 interact with the main chain carbonyl of Ile-130 and the side chain of Glu-87, respectively. Similarly, the carboxyl group of the sialic acid moiety and the hydroxyl group at C-4 of the galactose moiety are involved in water-mediated contacts, interacting with the main chain carbonyl of Leu-86. In addition, the Nacetylgalactosamine moiety of the acceptor interacts with various residues including Glu-123, Asn-127, and Arg-129 through bound water to further stabilize the acceptor binding.

Figure 4.1: Structural analysis of the ternary complex of Cst-II

(A) Overall structure of Cst-II Δ 32 in complex with donor analog (CMP) and the trisaccharide acceptor (Neu5Ac- α -2,3-Gal- β -1,3-GalNAc). The flexible lid domain (residues 155-188) is shown in magenta. (B) Acceptor binding site of mutants (N51A, Y81F) showed complete absence of the trisaccharide. Structures of wild type Cst-II Δ 32, N51A, and Y81F mutants are shown in blue, orange, and yellow, respectively. All three structures are highly homologous (root mean square deviation of ~0.4 Å on 243 C- α atoms). The trisaccharide acceptor was found only in the structure of wild type enzyme and shown as a reference molecule to indicate the acceptor binding site. (C) Interactions between the trisaccharide acceptor and active site residues. Electron density of the trisaccharide acceptor in a refined $2F_o-F_c$ map is contoured at 1.5σ . Dotted lines indicate hydrogen bonding. (D) Active site of Cst-II with the trisaccharide acceptor and modeled donor (CMP-3FNeu5Ac). Residues interacting with donor and acceptor are shown in cyan and blue, respectively. Tyr-185 is shown in orange. Carbon atoms are shown in green and yellow for the trisaccharide acceptor and CMP (or CMP-3FNeu5Ac), respectively. The label of Ser-53 has been omitted for clarity. Non-carbon atoms are colored according to atom type (nitrogen, blue; oxygen, red; phosphorus, orange; fluorine, silver).



4.3.4 Mutant Structures

Two essential residues (Asn-51 and Tyr-81) interact with the *N*-acetylamino group of the sialic acid moiety of the trisaccharide acceptor. Crystal structures of mutant forms at these positions, Cst-II Δ 32 N51A and Y81F, in the presence of CMP, shows the same overall tertiary structures as observed in the wild type structure (root mean square deviation of ~0.4 Å on 241 C- α atoms). Mutant crystals were grown in the same condition containing both CMP and the trisaccharide acceptor used for the crystallization of the wild type enzyme. However, the structures of these mutants show only electron density corresponding to CMP in the donor site with no visible density corresponding to the trisaccharide acceptor (Figure 4.1B). Both mutant structures show only the electron density of CMP without any density corresponding to the trisaccharide acceptor.

4.3.5 Kinetic Analysis

Kinetic analysis of wild type Cst-II Δ 32 and mutants were performed to characterize the nature of enzyme catalysis (Table 4.2). All kinetic experiments were carried out at 37 °C, and data were fitted to the Michaelis-Menten equation. For the wild type enzyme, both disaccharide (Gal- β -1,3-GalNAc- α -OBn) and trisaccharide (Neu5Ac- α -2,3-Gal- β -1,3-GalNAc- α -OBn) acceptor substrates were analyzed to compare kinetic characteristics towards two distinct acceptors. Kinetic characterizations for mutants (N51A, Y81F, and R129A) were performed with the trisaccharide acceptor.

Cst-II Variant	Acceptor	K_m (mM)	k_{cat} (min ⁻¹)	k _{cat} /K _m
wt (Δ32)	Galβ1,3GalNAcαOBn	34.7±4.0	7.9	0.2
wt (Δ32)	Neu5Aca2,3Galβ1,3GalNAcaOBn	6.9±0.4	61.1	8.9
Y81F	Neu5Aca2,3Gal	13.7±1.1	39.5	2.9
N51A	Neu5Aca2,3Gal	5.9±0.7	1.1	0.2
R129A	Neu5Aca2,3Galβ1,3GalNAcaOBn	18.6±3.3	2.8	0.2
N51A/Y81F ^a	Neu5Aca2.3GalB1.3GalNAcaOBn	-	-	-

Table 4.2: Kinetic parameters for acceptor substrates with wild type Cst-II Δ 32 and mutants.

^{*a*} Kinetic constants for N51A /Y81F double mutant could not be obtained due to the complete loss of activity

Cst-IIA32 showed significantly higher binding affinity and catalytic constant with the trisaccharide acceptor than those of the disaccharide acceptor. The K_m value for the trisaccharide was 6.9 µM, which is 5-fold lower than that of the disaccharide, suggesting that the enzyme preferably binds to the trisaccharide acceptor. Specificity constants (k_{cat}/K_m) for the trisaccharide and the disaccharide acceptors were 8.9 and 0.2 min⁻¹mM⁻¹, Comparison of specificity constants between two different acceptors respectively. revealed that Cst-IIA32 catalyzes the transfer of sialic acid more efficiently to the trisaccharide acceptor (Table 4.2). Kinetic characterizations of mutant enzymes revealed that all mutants significantly hamper the catalysis. Both N51A and R129A mutants showed dramatically reduced specificity constants (0.2 min⁻¹mM⁻¹). Y81F is the only mutant maintaining a comparable level of specificity constant (2.9 min⁻¹mM⁻¹). Dramatic reductions of k_{cat} values were observed in N51A and R129A mutant enzymes, implying that those residues are involved in the catalysis. Interestingly, Y81F mutant has catalytic efficiency (k_{cat} value 39.5 min⁻¹) comparable to that of the wild type enzyme (61.1 min⁻¹). Comparison of binding constants of Y81F (13.7 mM) and R129A (18.6 mM) mutants to

that of the wild type enzyme (6.9 mM) revealed that these mutations affect the acceptor binding. We could not measure kinetic constants for N51A/Y81F double mutant due to the completely abolished activity (Table 4.2).

4.4 Discussion

The bifunctional sialyltransferase Cst-II from the human pathogen *Campylobacter jejuni* serotype OH4384 catalyzes formation of an α -2,3-linkage to the O-3 of its galactose acceptor and subsequent α -2,8-linkage formation to O-8 of sialic acid of the initially formed sialoside product (Neu5Ac- α -2,3-Gal- β -1,3-GalNAc-R) in the lipooligosaccharide (LOS) at the outermost surface of the bacteria. Although the binary complex of Cst-II Δ 32 with the inert donor analogue CMP-3FNeu5Ac has revealed the molecular details of the nucleotide-sugar donor binding site, the lack of structural information on the binding of physiologically relevant acceptor sugars has hindered our ability to fully understand the necessary binding specificity and catalytic mechanism of this biologically important enzyme family. In this study, we provide the first structural analysis of acceptor-binding during the Cst-II-mediated sialylation of lipooligosaccharide in the food borne pathogen *Campylobacter jejuni*.

Initially, Cst-II Δ 32 was crystallized in the presence of the nonhydrolyzable donor analogue, CMP-3FNeu5Ac and the trisaccharide acceptor (Neu5Ac- α -2,3-Gal- β -1,3-GalNAc). However, only CMP-3FNeu5Ac was found in the active site with a completely closed lid domain. The acceptor bound ternary complex was obtained by cocrystallizations in the presence of another donor analogue, CMP and the trisaccharide acceptor. Structural superposition between the ternary complex of Cst-II Δ 32 and the previously reported donor complex showed no significant conformational differences in active site residues involved in the acceptor binding, suggesting the acceptor site is largely preformed once the nucleotide portion of the donor is bound to the enzyme.

Previously, extensive attempts to obtain the acceptor complex of Cst-II Δ 32 by using both cocrystallization and soaking with various acceptor analogues including lactose, 3'deoxy-lactose, and sialyllactose were unsuccessful (Chiu et al., 2004). The ternary complex can only be obtained by cocrystallization with donor analogue, CMP and the trisaccharide acceptor, Neu5Ac- α -2,3-Gal- β -1,3-GalNAc. Structural analysis of the trisaccharide acceptor binding features in the active site reveals that interactions between the *N*-acetylamino group of the sialic acid moiety of the acceptor and several key residues (Asn-51, Tyr-81, and Arg-129) are crucial for the proper binding of the trisaccharide acceptor substrate. Previously tried acceptor analogues such as lactose and 3'-deoxylactose do not contain the sialic acid residue carrying the important N-acetylamino group, thereby lacking the specificity for proper acceptor binding in the active site. In addition, lactose is a disaccharide that consists of galactose and glucose connected by a β 1,4-linkage, which is different from the β 1,3-linkage found in the natural disaccharide acceptor, Gal- β -1,3-GalNAc. Furthermore, lactose-based compounds do not contain the N-acetylamino group found in the GalNAc moiety of the natural acceptor, which potentially interacts with the flexible lid domain for proper orientation of the acceptor substrate during the catalytic reaction. Structural superimposition of the current ternary complex and the previously reported donor complex suggests that the Tyr-185 residue (Figure 4.1D), located in the lid domain, is a feasible candidate to interact with the *N*-acetylamino group of the GalNAc moiety of the acceptor.

The importance of key residues (Asn-51, Tyr-81, and Arg-129) localized in the acceptor-binding site was further validated by structural and kinetic analysis of mutants, showing a complete absence of the trisaccharide acceptor in active sites of mutant structures (Figure 4.1B) as well as a dramatic reduction of the specificity constants (Table 4.2). Interestingly, kinetic analysis on the Y81F mutant showed levels of catalytic efficiency comparable to that observed in the wild type enzyme (Table 4.2). Structural analysis of the acceptor-binding site identifies that the carbonyl of the *N*-acetylamino group of the sialic acid moiety in the trisaccharide acceptor interacts with Tyr-81 as well as an ordered water molecule. This water in turn directly interacts with the carbonyl of Ile-130 in the enzyme and the C-7 hydroxyl group of the acceptor to further stabilize acceptor binding, we believe potentially minimizing the adverse effects on acceptor binding imposed by loss of hydrogen bonding in the Y81F mutant form of Cst-II.

Extensive attempts to obtain the complex structure with the disaccharide acceptor (Gal- β -1,3-GalNAc) have been unsuccessful probably due to the weak affinity. Previously reported kinetic parameters of wild type Cst-II Δ 32 with lactose-based acceptor analogues demonstrated that lactose, which mimics the disaccharide acceptor, showed a 10-fold higher K_m value than that of the 3'-sialyllactose, an analogue of the trisaccharide acceptor substrate (Chiu et al., 2004). Similar kinetic behavior regarding acceptor binding was observed in the current kinetic analysis, in which the disaccharide acceptor shows 5-fold lower binding affinity compared to that of the trisaccharide.

Our structural and kinetic analysis of the acceptor bound ternary complex of Cst- $II\Delta 32$ coupled to the previously reported donor bound binary complex provide valuable insights into the catalytic mechanism of Cst-II during the α -2,8-sialyltransfer process. The closure of the flexible lid domain is known to be a required process for sialyltransfer, which protects the active site of the enzyme from bulk solvent, favoring electrostatic charges involved in the complex and minimizing undesirable side reactions such as hydrolysis of the donor substrate. Structural comparison between the donor complex and the trisaccharide acceptor complex shows that the catalytic base (His-188) is located at the ideal position to deprotonate the O-8 hydroxyl group of the sialic acid moiety of the trisaccharide acceptor substrate. The C-8 carbon of the sialic acid is approximately 4 Å away from both the imidazole side chain of His-188 and the anomeric carbon (C-2) of the sialic acid moiety of the donor substrate. Rotation of the hydroxyl group at the C-8 carbon of the sialic acid of the acceptor can change the distance even closer, providing optimally oriented acceptor substrate for the catalytic reaction. At the pH optimum of the Cst-II activity (pH 8), the imidazole side chain of His-188 is deprotonated and acts as the catalytic nucleophile to abstract proton from the O-8 hydroxyl group of the sialic acid of the acceptor. The acceptor substrate is properly oriented by interacting with key acceptor-binding residues including Asn-51, Tyr-81, and Arg-129 to facilitate this reaction step. Subsequently, the activated acceptor sugar directly attacks the anomeric carbon (C-2) of the sialic acid of the donor substrate, generating the oxocarbenium ion-like transition state followed by the release of the CMP facilitated by the interactions of Tyr-156 and Tyr-162 (Figure 4.2).



Neu5Ac- α -2,8-Neu5Ac- α -2,3-Gal-R'

Figure 4.2: Proposed mechanism of Cst-II showing all interacting residues for donor and acceptor substrates.

Donor substrate (CMP-Neu5Ac) is shown in green and acceptor substrate is shown in blue. Catalytic base (His-188) is shown in red.

Further features of the α -2,3-sialyltransfer process of Cst-II can also be rationally proposed by structural comparisons of the current ternary complex with the structure of Cst-I, a monofunctional α -2,3-sialyltransferase (Chiu et al., 2007). Essential residues in the acceptor binding site of the bifunctional Cst-II show high structural and sequence similarity to corresponding residues of the monofunctional Cst-I. For example, Asn-51, Ser-79, and Arg-129 residues in Cst-II are conserved in Cst-I as Asn-66, Ser-94, and Arg-144 respectively. Interestingly, Arg-144 is oriented approximately 1.4 Å closer to the acceptor binding site in the Cst-I structure as compared to its Cst-II Arg-129 counterpart, effectively creating a smaller active site pocket to accommodate its smaller disaccharide acceptor (Gal- β -1,3-GalNAc). We suggest this arginine residue also has a role to ensure the proper orientation of the disaccharide acceptor for the α -2,3- transfer reaction catalyzed by Cst-I and by analogy may play a similar set of acceptor site modulation in accommodating the smaller disaccharide acceptor in the α -2,3- sialyltransfer step of the bifunctional Cst-II. In Cst-I, the disaccharide acceptor can be further stabilized by a hydrogen bonding network involving Asn-166 and the carbonyl of Ser-94, and again by analogy via residues Asn-51 and Ser-79 in Cst-II. On the other hand, in the trisaccharide acceptor complex of Cst-II, Tyr-81 interacts with the N-acetylamino group of the sialic acid moiety of the trisaccharide acceptor. The corresponding residue (Phe-97) of Cst-I is unable to form an analogous direct interaction with the acceptor substrate suggesting this position is not critical to α -2,3- sialyltransfer in either Cst-I or by analogy, Cst-II.

The electrostatic surface map of Cst-II clearly shows the charge-complementarities between substrates and the active site of the enzyme (Figure 4.3A). In the structure of

LOS, the terminal oligosaccharide consists of a number of negatively charged sugar residues such as sialic acid, providing the specificity toward the positively charged active site of Cst-II. Furthermore, the highly positively charged cluster near the acceptor binding site highlights the potential of Cst-II in recognizing the negatively charged inner core oligosaccharide region of the LOS. The calculated molecular surface of Cst-II Δ 32 in combination with acceptor binding features obtained from structural analysis also suggest insights into how an oligomerized Cst-II tetramer binds to the core oligosaccharides of the LOS of *C. jejuni* and transfers sialic acids to specific saccharide units. The truncated C-terminal region of Cst-II is composed of 32 amino acids, which form a membrane spanning helix as predicted by various sequence-based algorithms (Cole *et al.*, 2008). This hydrophobic C-terminal region acts as a membrane-anchor to appropriately localize and potentially orient the enzyme to access the growing lipooligosaccharide chains for sialyltransfer reactions (Figure 4.3B).

Our structural and kinetic analysis of the ternary complex of sialyltransferase Cst-II with donor analogue and natural trisaccharide acceptor provides detailed acceptor binding features and further insights into the catalytic mechanism of this class of enzyme, thereby assisting the design of novel therapeutic agents to inhibit the sialylation of pathogenic bacteria.



Figure 4.3: Electrostatic surface map of the active site of Cst-II and proposed membrane-associated model of the Cst-II tetramer.

(A) The electrostatic molecular surface is shown in red for negatively charged region and blue for positively charged region. Both substrates (acceptor and donor) bind to the positively charged active site pocket of the enzyme. The yellow dotted-region indicates the potential binding site for inner core oligosaccharide units of the lipooligosaccharide. (B) Membrane-associated anchor domains were shown as green bars. Only two monomeric subunits of the Cst-II tetramer are represented for clarity (in blue and orange). Dotted lines indicate connecting loops between Cst-II Δ 32 and membrane-anchored domains. C-terminal helices of the Cst-II Δ 32 were shown in red. The flexible lid domain (residues 155–188) of the monomer is shown in magenta. Carbon atoms of the trisaccharide acceptor (Neu5Ac- α -2,3-Gal- β -1,3-GalNAc) and the nonhydrolyzable donor (CMP-3FNeu5Ac) are shown in green and yellow, respectively. Non-carbon atoms are colored according to atom type (nitrogen, blue; oxygen, red; phosphorus, orange; fluorine, silver). The precise membrane localization of Cst-II is unknown.

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<u>CHAPTER 5</u>: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Summary and Significance of Results

The main goal of this thesis was to analyze the structural and biochemical characteristics of enzymes directly involved in sialic acid metabolism in pathogenic bacteria. The work described in the preceding chapters in this thesis contribute to the current understanding of essential enzymes involved in the biosynthesis and post-synthetic process of sialic acid in bacterial pathogens, with particular focus on *N. meningitidis* and *C. jejuni*. Major contributions of the present thesis are highlighted and discussed below.

5.1.1 Design and Evaluation of the Inhibitor of Sialic Acid Synthase NeuB

Sialic acid synthase is the enzyme directly involved in the biosynthesis of sialic acid in pathogenic bacteria (Angata & Varki, 2002). It catalyzes the condensation reaction of *N*-acetylmannosamine (ManNAc) and phosphoenolpyruvate (PEP) to generate sialic acid (Tanner, 2005). This work (Chapter 2) describes the design and evaluation of the first potent inhibitor of bacterial sialic acid synthase NeuB from *N. meningitidis* (Liu *et al.*, 2009). The inhibitor was synthesized as a mixture of stereoisomers at C-2, which mimics the tetrahedral intermediate presumed to form in the catalytic reaction (Figure 2.1). Inhibition studies in combination with crystallographic analysis revealed that the inhibitor bearing (2*R*)-configuration is tightly bound to the active site of the enzyme. This suggests that the tetrahedral intermediate formed in the NeuB reaction also bears a (2*R*)configuration. Prior to this research work, the absence of structural information on the intermediate step of sialic acid synthase hindered the complete mechanistic understanding of this important enzyme in the biosynthesis of sialic acid. Together with structural and kinetic studies, a complete catalytic mechanism for NeuB is proposed (Figure 2.8). Furthermore, the current results also provide the evidence of the dual role of the metal cofactor (Mn^{2+}), which serves not only to activate the carbonyl of ManNAc in the first step of the reaction, but also to deliver hydroxide to the oxocarbenium intermediate in the second step of the reaction.

5.1.2 Structural and Biochemical Characterizations of Polysialic Acid *O*-Acetyltransferase OatWY

The neuroinvasive pathogen *Neisseria meningitidis* displays a polymeric sialic acidcontaining capsule that provides a means for the bacteria to evade the host's immune response during infection by mimicking host sialic acid-containing cell surface structures such as neural cell adhesion molecules (NCAM) (Kleene & Schachner, 2004, Virji, 2009). These capsules in certain serogroups of *N. meningitidis* including C, Y, and W-135 can be further acetylated by polysialic acid-specific *O*-acetyltransferases, a modification that correlates with decreased immunoreactivity and increased virulence (Lemercinier & Jones, 1996, Fusco *et al.*, 2007). Polysialic acid *O*-acetyltransferase OatWY from *N. meningitidis* serogroups Y or W-135 was first structurally and kinetically characterized as part of the present thesis work (Chapter 3) (Lee *et al.*, 2009). The previous lack of structural information on polysialic acid *O*-acetyltransferase from any bacterial species hindered our understanding of this important class of sialic acid-modifying enzymes. We reported the first structural and biochemical analysis of polysialic acid *O*-acetyltransferase OatWY from N. meningitidis serogroup Y in complex with either its natural donor (acetyl-CoA) or donor analogues (CoA, S-(2-oxopropyl)-CoA). We also reported the exquisite acceptor specificity of this enzyme that OatWY is highly specific for the unique form of the heteropolymeric polysialic acid (*N. meningitidis* Y polysaccharide, $[\rightarrow 6)$ -Glc-(α 1 \rightarrow 4)-Sia- $(\alpha 2 \rightarrow]_n)$, and does not catalyze the acetylation reaction on the homopolymeric polysaccharide of E. coli K1 or N. meningitidis serogroup B ($[\alpha(2\rightarrow 8)Sia]_n$). In addition, our mutagenesis studies in combination with structural analysis confirmed the role of two conserved residues (His-121 and Trp-145) in the catalytic mechanism, and further highlighted the significant side chain reorientation of Tyr-171 that blocks the active site of the enzyme in the apo structure. Collectively, our results revealed the first structural features of a bacterial polysialic acid O-acetyltransferase, and provided significant new insights into its catalytic mechanism and specificity for the capsular polysaccharide of serogroup Y meningococci. Deriving from the first structural characterization of polysialic acid O-acetyltransferase, the structure serves as a model for sialic acid-specific O-acetyltransferases pathogenic including of other bacteria group В Streptococcus, Campylobacter jejuni, and Escherichia coli K1. As acetylations on sialic acids of capsular polysaccharides make bacterial pathogens more virulent, understanding the structure and mechanism of the sialic acid-specific O-acetyltransferase is valuable to the development of antibacterial compounds.
5.1.3 Structural and Kinetic Characterizations of the Acceptor Complex of Sialyltransferase Cst-II

The sialylation of the lipooligosaccharide (LOS) outer core in bacterial pathogen C. *jejuni* is considered a major virulence determinant providing a protective barrier to evade host's immune system by mimicking the carbohydrate moieties of human gangliosides (Gilbert et al., 2002, Guerry et al., 2002). This molecular mimicry is also recognized as the triggering factor of the development of autoimmune diseases such as Guillain-Barré syndrome (Endtz et al., 2000). Sialyltransferases from this bacterial pathogen are responsible for the production of the sialylated lipooligosaccharide by transferring a sialic acid moiety from the donor substrate, CMP-Neu5Ac onto the terminal carbohydrate moiety The work presented as part of this thesis (Chapter 4) describes structural of LOS. characterizations of sialyltransferase Cst-II from C. jejuni in complex with its donor sugar analogue CMP and the terminal trisaccharide (Neu5Ac- α -2,3-Gal- β -1,3-GalNAc) of its natural acceptor. The absence of structural information on the acceptor binding mode hampered our ability to understand the substrate specificities and complete catalytic mechanism of this enzyme. Structural analysis of Cst-II in complex with the donor sugar analogue CMP and its natural acceptor provides valuable information on binding specificities of donor and acceptor substrates, thereby significantly enhancing our understanding mechanism sialyltransferases. Additional for the of kinetic characterizations of mutants of the acceptor binding residues provide the detailed binding mode of the acceptor, thereby assisting the design of potential antibacterial agents to inhibit the sialylation of pathogenic bacteria.

5.2 Future Directions

5.2.1 Preparation and Evaluation of Additional Intermediate Analogues for Sialic Acid Synthase NeuB

The preparation and direct stereochemical analysis of the tetrahedral intermediate formed in the sialic acid synthase reaction had been a challenging and difficult task due to the spontaneous collapse of the tetrahedral intermediate. In this thesis work, a stable deoxy analogue of the tetrahedral intermediate in absence of the C-2 hydroxyl group was synthesized and its stereochemical features were examined as discussed in section 2.3. The preparation and evaluation of additional phosphonate-based analogue retaining the C-2 hydroxyl group would further confirm the findings of the present studies (Figure 5.1). The phosphonate-based analogue successfully mimics the tetrahedral intermediate retaining the C-2 hydroxyl group, thereby providing direct evidence for the stereochemical basis of the tetrahedral intermediate.



Figure 5.1: Structure of the phosphonate-based analogue.

5.2.2 Ternary Complex of Polysialic Acid O-Acetyltransferase OatWY

Attempts to obtain both donor and acceptor substrates in the active site of polysialic acid *O*-acetyltransferase OatWY have been extensively tried. Various acceptor analogues have been used for co-crystallization and/or soaking experiments (Table 5.1). However, none of these candidates yielded a successful ternary complex structure of the enzyme presumably due to the poor affinity of the acceptor substrate ([Glc-(α 1 \rightarrow 4)-Sia] disaccharide) or the heterogeneous nature of the current acceptor (*N. meningitidis* Y polysaccharide, ([\rightarrow 6)-Glc-(α 1 \rightarrow 4)-Sia-(α 2 \rightarrow]_n)). Structural characterization of the acceptor binding mode would significantly enhance our understanding for mechanistic features and provide direct evidence for acceptor specificities in the active site of this enzyme.

Table 5.1: Attempts for obtaining ternary complex structure of OatWY.

Donor analogue	Acceptor analogues
CoA	Sialic acid (Neu5Ac)
	Disaccharide [Glc-(α 1 \rightarrow 4)-Sia]
	<i>N. meningitidis</i> Y polysaccharide (acetylated form) [Glc- $(\alpha 1 \rightarrow 4)$ -Sia(OAc)] _n
	<i>N. meningitidis</i> Y polysaccharide (deacetylated form) $[Glc-(\alpha 1 \rightarrow 4)-Sia]_n$
	Colominic acid $[\alpha(2\rightarrow 8)Sia]_n$

5.2.3 Disaccharide Acceptor Complex of Sialyltransferase from C. jejuni

Sialyltransferase Cst-II isolated from the strain OH4384 of C. jejuni is a bifunctional sialyltransferase, transferring sialic acid to the terminal galactose moiety of the lipooligosaccharide (LOS) with α -2,3-linkage, and subsequently transferring a second unit of sialic acid to the initially formed sialoside with α -2,8-linkage (Gilbert et al., 2002). The ternary complex reported in the present thesis described the structure in complex with the donor analogue CMP and the terminal trisaccharide of its natural acceptor, Neu5Ac- α -2,3-Gal- β -1,3-GalNAc (Chapter 4). This trisaccharide compound is the acceptor for α -2,8sialyltransferase activity of Cst-II. Because Cst-II is a bifunctional enzyme possessing both α -2,3- and α -2,8- sialyltransferase activities, attempts to obtain another ternary complex with the disaccharide moiety (Gal-β-1,3-GalNAc) of its natural acceptor in order to evaluate the difference in substrate specificities between α -2,3- and α -2,8sialyltransferase activities have been extensively tried. Various derivatives of the disaccharide acceptor have been used for co-crystallization and/or soaking experiments (Table 5.2). However, none of these attempts showed a ternary complex structure most likely due to the poor affinity of disaccharide acceptors.

Enzyme	Donor/Donor analogue	Disaccharide acceptor derivatives
Cst-II	N/A	Gal-β-1,3-GalNAc-α- <i>o</i> NP
	CMP-3FNeu5Ac	Gal-β-1,3-GalNAc-α- <i>o</i> NP
	CMP	Gal-β-1,3-GalNAc-α- <i>o</i> NP
	CMP	Gal-β-1,3-GalNAc-α- <i>p</i> NP
	CMP	Gal-β-1,3-GalNAc-α-OBn
Cst-II N51T & I53N	СМР	Gal-β-1,3-GalNAc-α- <i>p</i> NP
	CMP	Gal-β-1,3-GalNAc-α- <i>o</i> NP
	СМР	Gal- β -1,3-GalNAc- α -OCH ₂ CH ₂ N ₃

Table 5.2: Attempts for obtaining disaccharide acceptor complex for Cst-II.

A monofunctional Cst-II is another variant of sialyltransferase from C. jejuni serogroup O19 (Gilbert et al., 2002). This version of Cst-II has only α -2,3sialyltransferase activity, however, its amino acid sequence is almost identical (97% identity) compared to the bifunctional Cst-II from C. jejuni serogroup OH4384, which shows only eight residue difference. The majority of amino acid substitutions occurred in regions exposed on the protein surface, which is distantly located from the active site of the Among those eight different residues, three are located in the active site enzyme. including Asn51Thr, Ile53Asn, and Leu54Phe. Mutant enzyme of Cst-II serotype OH4384 bearing both Asn51Thr and Ile53Asn mutations in the active site was generated and the siayltransfer activity was examined by TLC analysis to confirm the monofunctionality of this O19-like Cst-II_{OH4384} enzyme. Various co-crystallization and/or soaking trials with the monofunctional mutant of Cst-II_{OH4384} were carried out to obtain the disaccharide acceptor However, these attempts haven't yet produced a complex structure (Table 5.2). disaccharide acceptor complex structure (Table 5.2). Additional mutation at position 54

(Leu54Phe) would potentially facilitate the disaccharide acceptor binding in the active site of the enzyme due to the complete mimic of the active site of a monofunctional Cst-II₀₁₉.

5.2.4 Structural Analysis of UDP-GlcNAc 2-epimerase

Hydrolyzing UDP-GlcNAc 2-epimerase is the enzyme that catalyzes the epimerization of UDP-*N*-acetylglucosamine (UDP-GlcNAc) to *N*-acetylmannosamine (ManNAc) as the first committed step in the biosynthesis of sialic acid in bacterial pathogens (Murkin *et al.*, 2004, Vann *et al.*, 2004). Previous biochemical studies in combination of mutagenesis suggested the catalytic mechanism and also identified essentially conserved residues (Asp-100, Glu-121, and Asp-131) involved in catalysis of the enzyme (Figure 1.6) (Murkin et al., 2004).

Extensive work has been carried out to analyze the structure of UDP-GlcNAc 2epimerase SiaA from *Neisseria meningitidis*. For obtaining the ideal condition for crystallization and diffraction analysis, various constructs were made in combination of multiple truncations and surface entropy reduction (SER) approaches (Table 5.3). The identification of mutation sites for the surface entropy reduction was performed by using the SER Prediction Server[•] (Goldschmidt *et al.*, 2007). The surface entropy reduction approach involves the selective replacement of bulky surface exposed and high entropy amino acids (e.g., Lys or Glu) with residues that have small and low entropy side chains such as alanines, thereby facilitating the formation of well-ordered protein crystals (Derewenda, 2004).

^{*} SER: Surface Entropy Reduction Prediction Server, http://nihserver.mbi.ucla.edu/SER/

Purification protocols were developed and various constructs were purified to homogeneity. Based on the results of static light scattering, SiaA and its mutants were found to form a tetramer in solution. Extensive crystallization screenings and subsequent optimizations were performed, however, these protein crystals only diffracted x-ray maximally to 3.5 Å, insufficient for subsequent structure analysis. Furthermore, various attempts to solve the protein structure including the soaking with heavy-atom derivatives or halides, MAD (Multiwavelength Anomalous Dispersion) phasing by selenomethionine (SeMet)-incorporated constructs, and multiple trials of molecular replacement were unsuccessful.

Construct	Solubility	Crystallizability	Diffraction
N-terminal Histidine tag	0	0	8.0 Å
C-terminal Histidine tag	Х	N/A	N/A
Surface Entropy Reduction	0	0	Х
Tagless	0	0	3.5 Å
-8/SiaA	Х	N/A	N/A
-14/SiaA	Х	N/A	N/A
-20/SiaA	Х	N/A	N/A
SiaA/-5 (±Refolding)	Х	N/A	N/A
SiaA/-10 (±Refolding)	Х	N/A	N/A
SiaA/-17	Х	N/A	N/A

Table 5.3: Constructs of UDP-GlcNAc 2-epimerase SiaA from N. meningitidis.

Additional crystallization screenings of the native SiaA in the presence of protease (chymotrypsin) were carried out and well-diffracting crystals were produced (1.8 Å). The structure of this protease-treated crystal was solved by halide soaking experiments, but

resulting structure only showed a partial structure (aa 180–333) of the enzyme, which was insufficient to analyze the overall architecture of the enzyme, including the active site residues (Asp-100, Glu-121, and Asp-131) located in the missing domain.

Several homologues of SiaA including NeuC from *E. coli* K1 (~30% amino acid sequence identity), NeuC from group B streptococcus (~25% amino acid sequence identity), and NeuC1 from *C. jejuni* (~40% amino acid sequence identity) were also cloned from genomic DNA, purified to homogeneity, and extensively screened with various conditions. One of these homologues, NeuC1 from *C. jejuni*, yielded a well-ordered protein crystal and an x-ray diffraction dataset has been collected to 2.7 Å. The SeMet-incorporated construct of NeuC1 enzyme were generated and further optimization of crystallization conditions is currently underway.

5.2.5 Outer Membrane Protein CtrA from N. meningitidis

Synthesized and polymerized sialic acids are transported from their cytoplasmic sites of synthesis to the surface of pathogenic bacteria across inner/outer membranes (Vimr & Steenbergen, 2009). Translocation of polysaccharides in such a complex biological system involves a variety of membrane-associated proteins. Human pathogen, *Neisseria meningitidis*, also contains a gene cluster responsible for the transport of polysaccharides to the extracellular matrix (Frosch *et al.*, 1989, Schoen *et al.*, 2009) (Figure 1.16). One of the gene products, CtrA, is an integral outer membrane protein uniquely expressed in *N. meningitidis*, and is highly conserved in all clinically important meningococcal serotypes (A, B, C, W-135, and Y) (Frosch *et al.*, 1992).

Work has been instigated for the structural characterization of CtrA. Full-length construct was generated, and growth conditions for protein overexpression in *E. coli* were optimized. A variety of detergents were screened to find the optimal type and condition required for the maximal solubility and stability of the protein. Purification protocol was developed, and the protein was purified to reasonable homogeneity. Currently the purification scheme was under further optimization to obtain the sample with higher purity and homogeneity. Based on the static light scattering experiment, CtrA was found to exist as a hexamer in solution. Crystallization screenings have been tried, and small crystals were observed. One of these CtrA crystals diffracted x-ray ~16 Å, which was insufficient for subsequent structure determination. Optimizations of purification procedures and crystallization conditions are currently underway.

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