CRYSTALLOGRAPHIC STUDIES OF BACTERIAL PROTEINS INVOLVED IN SIALIC ACID SYNTHESIS

By

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ABSTRACT

In *Neisseria meningitidis* and related bacterial pathogens, sialic acids play critical roles in mammalian cell immunity evasion, and are synthesized by a conserved enzymatic pathway which includes sialic acid synthase (NeuB, SiaC, or SynC). NeuB catalyzes the condensation of phosphoenolpyruvate (PEP) and N-acetyl mannosamine (ManNAc), directly forming *N*-acetylneuraminic acid (NeuNAc or sialic acid). Despite the important biological roles of sialic acids, very little is known about the structural and mechanistic properties of the enzymes which create them. This thesis primarily focuses on the determination of the first structure of a sialic acid synthase, that of NeuB, revealing a unique domain-swapped homodimer architecture consisting of a $(\beta/\alpha)_8$ barrel (TIM barrel) type fold at the N-terminal end, and a domain with high sequence identity and structural similarity to the ice binding type III antifreeze proteins at the C-terminal end of the enzyme. The structures of NeuB in the malate-bound form, bound to PEP and the substrate analog N-acetylmannosaminitol, and bound to the intermediate analogue N-acetylneuraminic borate were determined to 1.9, 2.2, and 2.2 Å resolution, respectively. Typical of other TIM barrel proteins, the active site of NeuB is located in a cavity at the C-terminal end of the barrel; however, the positioning of the swapped antifreeze-like domain from the adjacent monomer provides key residues for hydrogen bonding with substrates in the active site of NeuB, a structural feature which leads to distinct modes of substrate binding from other PEP utilizing enzymes which lack an analogous antifreeze-like domain. Our observation of a direct interaction between a highly ordered Mn$^{2+}$ and the N-acetylmannosaminitol in the NeuB active site also suggests an essential role for the ion as an electrophilic catalyst that activates the ManNAc carbonyl to the addition of
PEP. In addition, the development of a coupled assay to monitor NeuB reaction kinetics, and an $^{18}$O-labelling study that demonstrates the synthase operates via a C-O bond cleavage mechanism are discussed in order to further analyze structure-function relationships.
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I would like to thank my family and brothers for their support throughout my studies. This research would have not been possible if it were not for the talents and resources of my supervisor Dr. Natalie Strynadka. I am thankful for her limitless knowledge, scientific guidance, and patience with the completion of my research. I would also like to thank my supervisory committee members, Dr. Martin Tanner, Dr. Michael Murphy, and Dr. Lawrence McIntosh who have provided assistance and support for my work.

Past and present members of the Strynadka lab have provided a tremendous amount of technical and emotional support. Special thanks go to Dr. Andrew Lovering for teaching me everything about crystallography and knowing how to play a mean round of pitch and putt. I would like to thank Richard Pfuetzner for all his protein purification skills and light scattering handy work, and Dr. Gunnar Olovsson for keeping the X-ray machine in order. I must also thank Dr. Michela Bertero, Dr. Lodovica Loschi, Dr. Paula Lario, Tanya Hills, Liza deCastro, Calvin Yip, and Cecilia Chiu for all their technical assistance. Several aspects of this work were made possible through contributions from our collaborators, Dr. Martin Tanner and Dr. Warren Wakarchuk. David Simard in Dr. Tanner’s laboratory provided the kinetic and catalytic data, whereas Dr. Michel Gilbert and Dr. Scott Dick must be thanked for providing me with the initial NeuB clones. The technical assistance from the employees of beamlines 8.2.1 and 8.2.2 at the ALS in Berkley have also provided a tremendous amount of help.

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MANUSCRIPTS AND AUTHORSHIP

Much of the work presented in this thesis contains portions from a manuscript published in the Journal of Biological Chemistry [Gunawan, J., Simard, D., Gilbert, M., Lovering, AL., Wakarchuk WW., Tanner, ME., and Strynadka NCJ. Structural and mechanistic analysis of sialic acid synthase NeuB from Neisseria meningitidis in complex with Mn$^{2+}$, phosphoenolpyruvate, and N-acetylmannosaminitol. J. Biol. Chem. 2004 Oct. 29 (epub ahead of print)]. The manuscript was primarily written by myself and revised by my supervisor Dr. Natalie Strynadka. Dr. Martin Tanner prepared the kinetic and mechanistic information in the manuscript. All kinetic and mechanistic studies were done by David Simard in the laboratory of Dr. Tanner. Dr. Scott Dick in the laboratory of Dr. Warren Wakarchuk did the initial cloning of the NeuB constructs. Purification, crystallization, and structural determination of NeuB were done by myself.
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<table>
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<tr>
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<tr>
<td>A5P</td>
<td>D-arabinose 5-phosphate</td>
</tr>
<tr>
<td>AFP</td>
<td>Antifreeze protein</td>
</tr>
<tr>
<td>ALS</td>
<td>Advanced Light Source</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCD</td>
<td>charge coupled device</td>
</tr>
<tr>
<td>CMP-NeuNAc</td>
<td>cytidine-5'-monophospho-N-acetyl-neuraminic acid</td>
</tr>
<tr>
<td>CTP</td>
<td>cytidine triphosphate</td>
</tr>
<tr>
<td>DAH7PS</td>
<td>3-deoxy-D-arabino-heptulosonate synthase</td>
</tr>
<tr>
<td>DDT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E4P</td>
<td>D-erythrose 4-phosphate</td>
</tr>
<tr>
<td>ESMS</td>
<td>electrospray mass spectrometry</td>
</tr>
<tr>
<td>G3P</td>
<td>glyceraldehydes 3-phosphate</td>
</tr>
<tr>
<td>HCL</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HMM</td>
<td>high molecular mass</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KDO8PS</td>
<td>3-Deoxy-d-manno-octulosonate 8-phosphate synthase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani medium</td>
</tr>
<tr>
<td>LMM</td>
<td>low molecular mass</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight</td>
</tr>
<tr>
<td>ManNAc</td>
<td>N-acetyl mannosamine</td>
</tr>
<tr>
<td>rManNAc</td>
<td>reduced N-acetyl mannosamine (N-acetlmannosaminitol</td>
</tr>
<tr>
<td>NeuNAc</td>
<td>N-acetyl neuraminic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NeuNAcB</td>
<td>N-acetyl neuraminic borate</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>optical density at 600 nm wavelength</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>rmsd</td>
<td>root mean square deviation</td>
</tr>
<tr>
<td>SAD</td>
<td>Single wavelength anomalous diffraction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SeMet</td>
<td>selenomethionine</td>
</tr>
<tr>
<td>TIM</td>
<td>triose-phosphate isomerase</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>uridine 5'-pyrophosphate-N-acetylg glucosamine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VdW</td>
<td>van der waals</td>
</tr>
<tr>
<td>v/v</td>
<td>unit volume (mL) per unit volume (mL)</td>
</tr>
<tr>
<td>w/v</td>
<td>unit weight (g) per unit volume (mL)</td>
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Chapter 1: INTRODUCTION

1.1 The Biological Importance of Sialic Acids

The role of carbohydrates in biological systems is wide and varied as they not only function as the primary source of energy, through the form of starch or glucose, but they also play integral structural and metabolic roles as components of the cell membrane. Monosaccharides are the basic building blocks of complex carbohydrates and through a variety of glycosidic linkages, a vast array of polyhydroxyaldehydes and polyhydroxyketones are created. The most common monosaccharides found in higher animal organisms include hexoses (6 carbon neutral sugars), hexosamines (hexoses with an amine group) and sialic acids (9-carbon acidic sugars) (Varki, 1999). Sialic acids are said to be the most important sugar molecules in higher organisms due to their role in mediating diverse biological processes as structural components of glycoproteins and glycolipids (Schauer, 2000). This important monosaccharide was first discovered in bovine mucus and brain matter by Gunnar Blix in 1936 (Blix, 1936) and Ernst Klenk in 1941 (Klenk, 1941), respectively.

1.1.1 Biological Diversity in Mammalian Cells

Sialic acid (also referred to as neuraminic acid), derives from the Greek word sialon (saliva) in which they were first found. It is the general title given to a group of nine-carbon keto sugars derived from the parent compound 2-keto-3-deoxy-5-acetamido-D-glycero-D-galacto-nonulosonic acid (N-acetylneuraminic acid [NeuNAc]) (Vimr et al., 2004). These carbohydrates are found in a variety of organisms including higher animals (Varki, 1992), several types of pathogenic bacteria (Varki, 1992), insects (Schauer, 2004), and in cell cultures derived from Arabidopsis thaliana (Shah et al., 2003). In
mammalian species, sialic acids are generally observed as the terminal sugar residue at
the ends of oligosaccharide chains of cell surface glycoprotein and glycolipids (Varki,
1993). The presence of these carbohydrates on the external surface suggests its
importance in cellular interactions. Under general terms, the role of sialic acids in
mammalian cells can be classified into two groups: 1) acting as a biological target (ie. a
ligand), which interacts with a given receptor thus modulating certain processes (Schauer,
2000), and 2) acting as a biological mask, that is, representing a structural impediment to
a recognition site (Schauer, 1985).

The biological role of sialic acids in terms of participating in ligand-receptor
interactions ranges from development, recognition, activation (Schauer, 2000; Varki,
1999), and cell-cell adhesion among several types of blood cells (Brinkman-Van der
Linden and Varki, 2000; Kim et al., 1999; Norman et al., 2000). Cell recognition
processes regulated by sialic acids include i) mediation of glycoproteins/glycolipids and
cell membranes through charge-charge repulsion (sialic acids are negatively charged), ii)
controlling cellular regulation through acting as a chemical messenger, iii) regulation of
transmembrane receptors, iv) influencing membrane transport, v) mediation of the
lifespan of glycoproteins, and vi) influencing the selectivity of the endothelium
(Schauer, 2000). Initial experiments of the importance of sialic acids in blood cells
involved the discovery of sialic acid-binding lectins in macrophages of murine bone
marrow (Crocker et al., 1991). This "sialoadhesin" is a member of the immunoglobulin
superfamily (IgSF) (Angata and Brinkman-Van der Linden, 2002), which are responsible
for regulating the interaction of maturing blood cells (B-lymphocytes) with T-
lymphocytes in the development of the immune system (Bhunia et al., 2004; Crocker and Varki, 2001).

Besides binding to mammalian receptors, sialic acids also play a large role in acting as ligands for pathogenic and non-pathogenic viruses, bacteria and protozoa. The most well known interaction involves the binding of influenza A virus, through the hemagglutinin receptor, to human erythrocytes (Hirst, 1948). Binding of the virus leads to release of the sialic acid hydrolyzing enzyme neuraminidase inducing subsequent spread of the infection. Structural studies of this enzyme have led to the production of the sialidase inhibitor, Zanmavir, used for the treatment of influenza. Other viruses which bind via sialic acids include corona, polyoma and HIV (Tsai et al., 2003).

In contrast to the function of sialic acids as ligands for receptors, these carbohydrates also play an important role as biological masks. Two examples of this role include, the masking of penultimate sugars (ie. galactose) that are to be recognized by receptors, and the shielding of antigenic sites found in macromolecules (Schauer, 2004). In the first example, desialylation allows for the recognition of galactose-specific lectins, leading to the transfer of certain cells to specific sites. This targeting of particular molecules generally leads to the regulation of degradation, whether it is a physiological or pathological situation. Red blood cells, exposed to sialidases and thus demasking galactose residues, bind to galactose-specific receptors of phagocytes and are taken up and degraded (Muller et al., 1983). Malignant cells can be removed in a similar situation; however, hyper-sialylation can in turn protect these cells from humoral defense systems. Sialic acids are recognized by host-cells as “self”, thereby preventing recognition by the immune system. Loss of the carbohydrate on the surface of cells, changes their identity
to “non-self”, thereby leaving them susceptible to innate immunity. It can be said that these monosaccharides are a part of the innate immunity system (Schauer, 2004). The loss of sialic acids through bacterial or viral sialidases exposes cellular antigens, leading to the production of auto-antibodies. This example shows the importance of sialic acids in hiding antigenic determinants that would normally spark an innate immune response.

1.1.2 Sialylation in Pathogenic Bacteria

Due to the biological importance of sialic acids in higher organisms, certain types of pathogenic bacteria have exploited this observation to their advantage. *Campylobacter jejuni*, *Escherichia coli*, and *Neisseria meningitidis* are examples of pathogenic bacteria that produce sialic acid via an enzymatic pathway that differs from that of eukaryotes (Vimr et al., 2004), whereas other types of bacteria (*Neisseria gonorrhoeae*) obtain sialic acids through scavenging sialyl precursors from mammalian hosts (Parsons et al., 1988). Bacteria also use host sialic acids as a source of carbon, nitrogen, energy and amino acids for cell wall production (Plumbridge and Vimr, 1999). The presence of sialic acids on the surfaces of these pathogens leads to a variety of processes, with the most important being resistance to innate immunity responses by the host organism (see Table 1.1.2.1 – adapted from Roberts, 1996).

Table 1.1.2.1 Functions of Sialic Acids on Pathogenic Bacteria

<table>
<thead>
<tr>
<th>Function</th>
<th>Relevance</th>
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<tr>
<td>Prevention of desiccation</td>
<td>Transmission and survival</td>
</tr>
<tr>
<td>Adherence</td>
<td>Colonization of oral surfaces</td>
</tr>
<tr>
<td></td>
<td>Colonization of indwelling catheters</td>
</tr>
<tr>
<td></td>
<td>Bacteria-plant interactions</td>
</tr>
<tr>
<td>Resistance to non specific host immunity</td>
<td>Complement-mediated phagocytosis</td>
</tr>
<tr>
<td></td>
<td>Complement-mediated killing</td>
</tr>
<tr>
<td>Resistance to specific host immunity</td>
<td>Poor antibody response to the capsule</td>
</tr>
</tbody>
</table>
Sialic acids are integrated into bacterial cells under two scenarios: 1) as a series of repeating subunits forming a polysialic acid (PSA) capsular polysaccharide, and 2) as single units attached to the terminal ends of lipooligosaccharides (LOS) present on the surface of the outer membrane of bacterial cells (Vimr et al., 2004) (see Fig. 1.1.2.1)

Although capsular polysaccharides can consist of other monosaccharides that would cause an immune response, those that contain NeuNAc, such as *E. coli* K1 or *N. meningitidis* serogroup B are poorly immunogenic (Bhattacharjee et al., 1975). Both organisms produce NeuNAc and the PSA capsule in a similar manner, with much of the understanding of capsular biosynthesis coming from studies of group B *meningococci* (Azuma et al., 2000). The expression of these capsules elicits a resistance to a host's specific immune response, through mounting a poor antibody response, due to structural
similarities between bacterial PSA capsules and polysaccharides on the host organism (Finne, 1982).

Pathogenic bacteria confer resistance to nonspecific host immunity through the actions of the PSA capsule and the single NeuNAc residues on the cell surface (Roberts, 1996). Upon the introduction of a "foreign" object, the host organism would normally mount a non-specific defense mechanism that involves the activation of the complement cascade via the alternative pathway. The initial step of the pathway involves binding of the protein C3b to the bacterial cell surface. Attachment of C3b leads to the binding of several other proteins in an activation cascade, including the attachment of the membrane attack complex on the bacterial outer membrane, resulting in lysis and death (Frank et al., 1987). Fig. 1.1.2.2 is a representation of the modes of resistance by PSA capsules in pathogenic bacteria.

![Fig. 1.1.2.2: Serum resistance to nonspecific immunity of PSA capsules. Figure adapted from Vogel and Frosch, 1999 (Vogel and Frosch, 1999). Sialic acid on LOS inhibit binding of C3b, whereas PSA capsules inhibit MAC insertion. SA, sialic acid; OMP, outer membrane protein; OM, outer membrane; MAC, membrane attack complex.](image)

The polysaccharide provides a barrier such that complement binding proteins are unable to bind the bacterial cell surface, preventing the activation of the alternative pathway. Single sialic acid molecules attached to the LOS on the bacterial outer membrane, appear
to bind C3b serum protein (Fig. 1.1.2.2), thereby breaking the complement activation cascade (Frank et al., 1987). Studies done with unencapsulated bacteria, but with a partially sialylated LOS, showed C3b binding inhibition, but near wild-type levels of nonspecific immunity response due to the lack of inhibition of MAC binding. Therefore, encapsulation prevents the binding of the MAC into the bacterial cell surface (Fig. 1.1.2.2) (Vogel et al., 1997). Additional direct methods by which capsular PSA inhibit the activation of the alternative pathway include inhibiting the binding of factor H (Michalek et al., 1988) and effecting the release of cytokine molecules (Cross, 1990). Factor H normally acts as a co-factor to enhance the binding of factor I to C3b, leading to the formation of the MAC. Indirectly, the highly negative charge of PSA capsules also leads to a greater degree of resistance to phagocytosis (Moxon and Kroll, 1990).

1.1 Structural Diversity of Sialic Acids

As mentioned in section 1.1, sialic acid is the common name given to approximately 50 distinct members of this monosaccharide family that are derived from the parent sugar molecule NeuNAc. The different members of the sialic acid family carry various constituents at the amino or hydroxyl groups (Fig. 1.2.1) (Schauer, 2000; Schauer, 2004; Varki, 1992).
Fig. 1.2.1: The Sialic Acid Family. Figure taken from (Schauer, 2004). Information regarding the nomenclature, abbreviations, and references can be found in (Angata and Varki, 2002; Li et al., 2003). Residues on the core sugar molecule can be linked depending on the species.

All non-glycosidic hydroxyl groups present in the structure are susceptible to acetylation, while the amino group is either acetylated or glycolylated. O-9 is the most common site to observe diverse modifications including acetylation, lactylation, and phosphorylation. Generally, only one O-acetyl group is observed, and usually at position O-9 (Schauer, 2004). The most recently discovered derivative of NeuNAc is the 1-tauryl structure found in the brain tissue of individuals diagnosed with Tay-Sachs disease (Li et al., 2003). All of these side chains can be present in different combinations, thus leading to the diversity of observed sialic acid residues in higher organisms. In addition, anhydro- and lactone forms, and 2-keto-3-deoxy-nonoic acid ([Kdn] absence of nitrogen group at C-5) have been identified in a number of microorganisms and animals.

The most common sialic acid found in nature is NeuNAc, with N-glycolylneuraminic acid (NeuNGc) and N-acetyl-9-O-acetyleneuraminic acid (Neu5,9Ac2) being the next most frequently encountered member of the family (Schauer,
NeuNGc, which is found commonly found in higher animals, including apes, but has no significant presence in healthy human cells, is the second most well studied sialic acid next to NeuNAc. The absence of this particular derivative in humans has been thought to be a result of a gene mutation (Irie et al., 1998). Presence of NeuNGc in humans can be attributed to tumors; however, the methods by which they are produced are still in question (Schauer, 2004).

O-acetylated sialic acids, at C-9, are also commonly found in humans and some bacterial organisms, whereas 4-O acetylation have been identified in single species within the animal kingdom (ie. horses) (Varki, 1992). Very limited information regarding the enzymes that are involved in their creation is available. The same goes for the less frequently observed derivatives of the sialic acid family: 9-O-lactylation, and 8-O-sulfation. However, a few studies involving the isolation of the enzyme involved in the creation of 8-O-methylation from the gonads of Asterias rubens have been carried out (Kelm et al., 1998). No one cell or organism has been known to contain all types of sialic acids. It appears as though the presence of certain sialic acids depends on the animal or cell species, in addition to the specific function of the cell (Schauer, 2004).

Sialic acids are generally found in glycosidic linkages of oligosaccharides, polysaccharides (including the PSA capsules in bacteria), glycoproteins, gangliosides, and lipopolysaccarhides (Varki, 1992). These linkages are created by sialyltransferases that join the C-2 donor hydroxyl to an acceptor hydroxyl group (Roseman, 2001). Since there are several hydroxyls available for linkage, and the number of sialic acids hovers at ~50, numerous combinations of oligosaccharides can be created. The most common isomer of sialic acid that exists in the free form is the β conformation (Fig. 1.2.1), which
lies axial to C2. This configuration exists in thermodynamic equilibrium with the minor \( \alpha \) isomer (Vimr et al., 2004). The formation of sialic acids and their transfer to the outer surface of the cell membrane constitutes a complex pathway.

1.3 Biosynthesis of Sialic Acids

The production of sialic acid in mammals and bacteria is a complex process involving several enzymes and coordinated within different compartments of the cell (in mammalian systems). Production of the enzyme in both types of cells occurs in a relatively similar manner, with some differences spread out through various stages.

1.3.1 Mammalian Production of Sialic Acid

Fig. 1.3.1.1 is an overview of the metabolism of sialic acid in the mammalian cell. The production and breakdown of the monosaccharide occurs in different compartments of the cell. Within the cytoplasm of the cell, the initial stages of sialic acid production involve the conversion of simple monosaccharides to the biologically important sugar Uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc). This activated sugar residue is then converted to N-acetylmannosamine-6-phosphate (ManNAc-6-P). NeuNAc is synthesized from the condensation of ManNAc-6-P and phosphoenolpyruvate through the actions of sialic acid synthase (Traving and Schauer, 1998). Following dephosphorylation of NeuNAc-9-P, cytidine monophosphate-NeuNAc synthase (CMP-NeuNAc) catalyzes the transfer of CMP from cytidine triphosphate (CTP) to the substrate within the nucleus of the cell. This activated sugar molecule is then transferred to the golgi apparatus where it can be transferred to existing glycoconjugates through the actions of sialyltransferases (Kelm and Schauer, 1997; Schauer, 1991). There are about 15 members of the sialyltransferase family that are known to create \( \alpha 2 \rightarrow 3, \alpha 2 \rightarrow 6, \alpha 2 \)
→8, and $\alpha 2 \rightarrow 9$ linkages between NeuNAc and respective accepting sugar (Traving and Schauer, 1998). Prior to attachment to a glycoconjugate, the $N$-acetyl group of CMP-NeuNAc can be modified through hydroxylation, thus creating CMP-NeuNGc in the cytosol (Schauer et al., 1995). All other modifications, such as $O$-acetylation and $O$-methylation, are done following binding to the glyconjugate, and before it is transferred to the cell surface.

Fig. 1.3.1.1: Production of sialic acid in the eukaryotic cell. Each enzymatic reaction is shown in their respective cellular compartment. See text for further details. Figure taken from Traving and Schauer (Traving and Schauer, 1998).
Catabolism of sialic acid residues are generally done by sialidases. Both serum sialoglycoconjugates and surface exposed sialic acid residues are susceptible to membrane-bound sialidases. Sialoglycoconjugates present in the cytosol are taken up by receptor mediated endocytosis in higher eukaryotes. Following fusion with a lysosome, sialic acids on the terminal ends of the glycoconjugate are detached by lysosomal sialidases. However, bacterial sialidases generally function on cell surface sialic acid residues as they are more readily exposed in the environment. NeuNAc and NeuNGc serve as good substrates for sialidases; however, O-acetylated sialic acid residues must first be exposed to sialate-O-acetyl esterases to remove the O-acetyl group. Following their release, free sialic acids are transported from the lysosome into the cytosol where they are either recycled for transfer onto another available glycoconjugate or they are degraded to free ManNAc and pyruvate. Acylneuraminate lyase, which catalyzes the aforementioned reaction, is also present in several bacterial species (Traving and Schauer, 1998).

1.3.2 Bacterial Production of Sialic Acid

The production of sialic acid in microorganisms follows a similar path to that of higher order eukaryotes. Studies of sialic acid metabolism in microorganisms has generally focused on the pathways from *E. coli* and *N. meningitidis*, which produce identical PSA capsules (Barry and Goebel, 1957; Vimr et al., 1995). As exemplified in fig. 1.3.2.1, the first committed step of the pathway involves conversion of UDP-GlcNAc to ManNAc by NeuC (*E.coli*) via an intermediate similar to the mammalian enzyme (Chou et al., 2003). The *N. meningitidis* orthologue to NeuC (also called SiaA) is thought
to produce ManNAc-6-P, which is then dephosphorylated to produce ManNAc (Petersen et al., 2001).

Fig. 1.3.2: Proposed model of sialic acid synthesis and degradation in E. coli. Figure taken from (Ringenberg et al., 2001). A similar pathway exists for N. meningitidis. Reactions that take place intercellularly are indicated within the rectangle representing the bacterial cell. See text for further details.

Following the production of ManNAc, sialic acid synthase, or NeuB (also called SiaC, SynC), catalyzes the condensation of ManNAc and PEP to produce NeuNAc. This step in the mechanism differs from that observed in the mammalian system, as it is the 6-phosphate derivative of ManNAc that reacts with PEP to produce NeuNAc-9-phosphate, which is then modified by a phosphatase to produce NeuNAc. The other known difference between the two pathways is the fact that in bacteria, the genes NeuC and
ManNAc kinase are organized into distinct synthetic and catabolic operons, respectively (Ringenberg et al., 2001).

Bacterial and mammalian cells also contain a reversible aldolase, NanA, which can produce sialic acid; however, this enzyme favours the breakdown of NeuNAc to its individual components ManNAc and PEP (Comb and Roseman, 1960). Similar to the mammalian pathway, free sialic acid in bacteria is activated by CMP-sialic acid synthetase (NeuA), which is the donor of sialic acid for sialyltransferases (NeuS). Bacteria also contain an enzyme called NeuD; however, its exact function has yet to be determined. The enzyme is required for sialic acid synthesis (Daines et al., 2000), and it is postulated to stabilize NeuB through direct hetero-oligomerization in E. coli (Daines and Silver, 2000). The expression of the PSA capsule following the actions of the sialyltransferases, requires yet another specific enzyme called NeuE (Vimr et al., 2004). NeuE contains a C-terminal binding domain that is thought to bridge PSA biosynthesis with export by binding to the inner membrane of the bacterial cell (Steenbergen et al., 1992).

1.4 The Role of Sialic Acid Synthase: A Closer Look

As mentioned in the previous section, bacterial sialic acid synthase, NeuB, catalyzes the condensation of ManNAc with PEP, directly producing sialic acid. On the other hand, the mammalian counterpart uses the 6-phosphate derivative of ManNAc to produce NeuNAc-9-phosphate. Fig. 1.4.1 exemplifies the key differences between bacterial and mammalian production of sialic acid at this particular stage.
The genes which code for the enzymes involved in PSA capsular biosynthesis in *E. coli* (and similarly *N. meningitidis*), are found in the 17-kb *kps* gene cluster (Silver et al., 1984; Vimr et al., 1989). This gene cluster is divided into three regions numbered sequentially (1-3). Region 1 contains genes which encode proteins that are involved in polymer transport across the outer membrane (Pazzani et al., 1993; Wunder et al., 1994), whereas region 3 contains genes that are thought to be involved in the energy-dependent transport of PSA across the cytoplasmic membrane (Pavelka et al., 1991). The genes in region 2 of the cluster include, *neuD*, *neuB*, *neuA*, *neuC*, *neuE*, and *neuS*, all of which produce respective enzymes involved in the synthesis, activation, and polymerization of sialic acid (the genes for *N. meningitidis* are also referred to as *sia*) (Boulnois and Jann, 1989). The *neuB* gene encodes for the 39 kDa sialic acid synthase, NeuB (Annunziato et al., 1995).
NeuB falls into a class of PEP-utilizing enzymes that form keto acids from a monosaccharide and PEP. The majority of mechanisms proposed for the various PEP-utilizing synthases imply that the reactions proceed via an open chain form of the sugar (Asojo et al., 2001; Furdui et al., 2004; Howe et al., 2003; Kaustov et al., 2003; Konig et al., 2004; Liang et al., 1998; Shulami et al., 2004; Shumilin et al., 2004; Wang et al., 2001), suggesting that NeuB may function in a similar manner. Fig. 1.4.2 illustrates the reaction catalyzed by NeuB from \textit{N. meningitidis}.

\begin{center}
\includegraphics[width=\textwidth]{neub_reaction.png}
\end{center}

\textbf{Fig. 1.4.2.}: Reaction catalyzed by \textit{N. meningitidis} sialic acid synthase (NeuB). NeuB catalyzes the condensation of PEP with ManNAc creating sialic acid and orthophosphate. Inset shows the structure of reduced ManNAc (rManNAc or N-acetylmannosaminitol), an unreactive linear substrate analogue.
According to the reaction illustrated in Fig. 1.4.2 a covalent bond is formed between the vinylic carbon, C-3, of PEP and the carbonyl group, C1, of ManNAc, followed by the release of hydrogen phosphate and a water molecule. The linear form of the sugar molecule then spontaneously cyclizes giving the physiological form of NeuNAc.

Two general mechanisms may be invoked for the action of sialic acid synthase, one employing C-O bond cleavage and the other employing P-O bond cleavage. Most enzymatic reactions involving PEP as a substrate generally proceed through a P-O bond cleavage; however, four particular enzymes proceed through a less energetically favourable C-O bond cleavage: 3-Deoxy-d-manno-octulosonate 8-phosphate synthase (KDO8PS), 3-deoxy-d-arabino-heptulosonate synthase (DAH7PS), UDP-GlcNAc enolpyruvyl transferase (MurZ), and 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS) (Walsh et al., 1996). Experiments with KDO8PS and DAH7PS indicate that the C-3 carbon of PEP adds to the carbonyl of ManNAc and water adds to the C-2 position of PEP to give a tetrahedral intermediate (Fig. 1.4.3) (Asojo et al., 2001; Furdui et al., 2004; Howe et al., 2003; Kaustov et al., 2003; Liang et al., 1998; Shulami et al., 2004; Shumilin et al., 2004; Wang et al., 2001). This would likely occur in a step-wise fashion involving an oxocarbenium ion intermediate. The tetrahedral intermediate would then lose phosphate to give the open chain form of NeuNAc that would spontaneously cyclize to the pyranose form in solution. A key experiment performed on the KDO8PS and DAH7PS reactions was to demonstrate that when [2-\textsuperscript{18}O]-PEP was used as a substrate, the \textsuperscript{18}O-label was found exclusively in the recovered orthophosphate (DeLeo et al., 1973; Hedstrom and Abeles, 1988). This is only consistent with a C-O bond cleavage.
mechanism (see labeled atoms in Figure 1.4.3). Despite this precedence, chemical intuition may lead one to favor a P-O cleavage mechanism in which water attacks the phosphate of PEP and liberates the enolate anion of pyruvate (not shown). The enolate then adds to the aldehyde of ManNAc to give the open-chain form of Neu5Ac. In this case, the use of [2-18O]-PEP would lead to label residing in the product and the formation of unlabeled orthophosphate. Enzymes shown to function through a P-O cleavage mechanism include pyruvate kinase (Seeholzer et al., 1991) and PEP carboxykinase (Matte et al., 1997). The carbon-carbon bond-forming step of such a reaction would resemble that catalyzed by N-acetylneuraminic acid lyase, a Type I aldolase that catalyzes the reversible cleavage of NeuNAc into pyruvate and ManNAc (Barbosa et al., 2000).

![Chemical reaction intermediates](image)

**Fig. 1.4.3:** The proposed C-O bond cleavage mechanism for NeuB. 18O-isotopic labels are included to highlight the fate of the bridging phosphate oxygen of PEP during catalysis.

### 1.5 PEP-Utilizing Enzymes and Antifreeze Proteins

As mentioned earlier, sialic acid synthase belongs to a family of PEP-utilizing enzymes whose members have been studied extensively, both mechanistically and
structurally. Earlier studies suggest that NeuB has several similarities and differences with these enzymes. In addition to similarities with PEP-utilizing enzymes, previous sequence analysis studies have suggested NeuB shares a curious relationship with a class of proteins that participate in the binding of ice and the prevention of its growth.

1.5.1 DAH7P and KDO8P Synthase

Two enzymes, 2-keto-3-deoxy d-manno-octulosonate 8-phosphate synthase (KDO8PS) and 2-keto-3-deoxy d-arabino-heptulosonate 7-phosphate synthase (DAH7PS), are known to utilize PEP to form complex sugar molecules. Both enzymes possess significant structural and mechanistic similarities. KDO8PS catalyzes the condensation of PEP with d-arabinose 5-phosphate (A5P) to form KDO8P (Levin and Racker, 1959), which is then dephosphorylated to produce KDO. KDO is an 8-carbon sugar that is an important component of LPS, binding lipid A with the O-antigen, present in all Gram-negative bacteria. Two classes of KDO8P synthase exist, Class I are metal-independent enzymes (E. coli), whereas Class II are metal-dependent enzymes (H. pylori) (Shulami et al., 2004). On the other hand, DAH7PS catalyzes the reaction between PEP and d-erythrose 4-phosphate (E4P) to form DAH7P (DeLeo and Sprinson, 1968). DAH7P is the initial substrate for the formation of aromatic amino acids, as well as other aromatic compounds involved in primary and secondary metabolism (Bentley, 1990). There are also two distinct classes of this particular enzyme: Class I DAH7P synthases are found in bacterial organisms and are homologous to the KDO8P synthases, whereas Class II enzymes are found in higher plants and are involved in secondary metabolism processes in microbial organisms (Birck and Woodard, 2001). The Class I DAH7P synthases also have a divalent metal requirement.
Extensive information regarding the structural and mechanistic properties of KDO8PS (Asojo et al., 2001; Radaev et al., 2000; Shulami et al., 2004) and DAH7PS (Furdui et al., 2004; Konig et al., 2004; Shumilin et al., 2004) is available. A 2.2 Å resolution crystal structure of T. maritima DAH7PS in complex with PEP, Cd^{2+}, and E4P was recently solved; however, the sugar substrate was observed in an apparently non-productive orientation (Shumilin et al., 2004). Both KDO8PS and DAH7PS exist as a homotetramer, with each monomer exhibiting an eight-stranded parallel β-barrel fold surrounded by eight helices ((β/α)_8 barrel) (Fig. 1.5.1.1) (Radaev et al., 2000; Wagner et al., 2000), a structure exemplified in triose-phosphate isomerase (TIM) (Banner et al., 1975)
As very little information regarding the structure or mechanism of sialic acid synthase has been available, and given the very low sequence identity with the characterized DAH7PS and KDO8PS (<10% identity for the \textit{E. coli} enzymes), it was not clear if the sialic acid metabolizing enzymes would adopt a similar fold and active site architecture.
1.5.2 Antifreeze Proteins

Antifreeze proteins (AFP) and glycoproteins (AFGP) are a special class of proteins found in a variety of organisms (fish, insects, and plants) that play an important role in binding ice and inhibiting its growth (Davies and Sykes, 1997). There are four known classes of antifreeze proteins (numbered sequentially I-IV) and a single group of antifreeze glycoproteins (Davies and Sykes, 1997) found in fish. Each of these classes contains proteins that are sequentially and structurally different. AFPs from plants and insects are equally diverse (Hon et al., 1995). The variety of AFPs that exist in nature have allowed various organisms to survive in harsh environments, thereby leading to species diversity. A rapidly evolving climate in nature could have propagated the expansion of the AFP structure, leading to its observed distribution amongst various species. The ice-binding mechanism is thought to be similar in both fish and insects, with interactions heavily based on hydrophobic and van der Waals bonds.

X-ray structures of the fish antifreeze proteins from type I (alanine-rich α-helix), II (C-type lectin fold), and III (globular, short β-strands) have been solved. The type IV structure is predicted to exist as a helix-bundle; however, no 3D structure has been determined for this particular protein (Jia and Davies, 2002). Over the past couple of years a growing list of AFP homologues that interact with sugars and polysaccharides has been produced. For example, type I and Ca²⁺ dependent lectins; type IV and Lipoprotein domain (Davies and Sykes, 1997). A sugar binding homologue to the type III AFP found in eel pouts was first suggested by Baardnsnes and Davies when sequence alignments showed a high degree of similarity between the fish type III AFP and the C-terminal region of mammalian sialic acid synthase (Baardsnes and Davies, 2001). The structure of
the fish type III AFP consists of 65 residues in a compact globular fold forming short β-strands that create a flat ice-binding surface (fig. 1.5.2.1) (Jia et al., 1996).

Fig. 1.5.2.1: 3D structure of the fish type III AFP at 0.62 Å. The overall structure of the ice-binding protein consists of a compact globular fold divided into two motifs. Each motif consists of a “pretzel fold” with four short β-strands and a 3₁₀-helix (Ko et al., 2003). Helices and loops are in yellow, strands are orange.

A homology model of the C-terminal domain of human sialic acid synthase was generated based on the structure of the fish type III AFP. Of the 16 core residues that have been identified as composing the ice binding face in type III AFPs, 11 were shown to be identical in human sialic acid synthase (Baardsnes and Davies, 2001).

Mammalian and bacterial sialic acid synthases have a relatively low sequence identity (<30%, data not shown), therefore a 3D structure of the enzyme would be required to observe any similarities with the type III AFPs. In addition, if NeuB does in fact contain a homologous antifreeze-like domain, it would be interesting to note the structural or enzymatic importance, if any, for the presence of such a domain.
1.6 Thesis Objectives

In light of the increasing occurrence of antibiotic resistance among pathogenic bacteria, there lies a need in developing novel antibiotic reagents that target areas that have not been previously utilized. In order to improve upon the decreasing effectiveness of current antibiotics, a detailed molecular understanding of target pathways is required. The importance of sialic acids in pathogenic bacteria in order to subvert mammalian host defenses, make their catalytic mechanism an attractive target for the production of new antibiotic compounds. The overall aim of this thesis is the structural characterization of proteins involved in the bacterial sialic acid synthesis pathway, more specifically that of sialic acid synthase NeuB (SiaC, SynC) from \textit{N. meningitidis}. NeuB catalyzes the condensation of PEP and ManNAc, directly forming sialic acid. The 3D structure of NeuB bound to its substrates and substrate analogues would provide information regarding important residues required in sugar binding and potential catalytic acids and bases that would drive the enzymatic reaction. In cooperation with David Simard in the laboratory of Dr. Martin E. Tanner of the Department of Chemistry, kinetic and mechanistic analysis of NeuB was performed to further support the structural data provided in this thesis. By analogy the work on NeuB also provides important new insights into the structure/function of mammalian sialic acid synthases, for which no direct structural information is currently available.

Further to the determination of the x-ray crystal structure of NeuB, a structural analysis of the enzyme with other PEP utilizing enzymes and the type III antifreeze proteins was carried out in order to highlight similarities and differences between the various proteins. Information from structural homologues would provide further
functional and mechanistic clues as to the specific action of NeuB in the essential formation of sialic acid.

It should be noted that the data provided in this thesis was published in the Journal of Biological Chemistry (Gunawan et al., 2004).
Chapter 2: MATERIALS AND METHODS

2.1 Reagents

All chemicals used for DNA manipulation, protein purification, analysis, and manipulation and crystal growth were purchased from commercial sources and were of reagent grade.

2.2 Expression Constructs

Wild type NeuB proteins were expressed from pCWori+ based constructs kindly provided by Dr. Michel Gilbert and Dr. Warren W. Wakarchuk in the Institute for Biological Science, National Research Council of Canada. Initial cloning of NeuB was done by Dr. Scott Dick in the aforementioned laboratory. The NeuB plasmid encodes for ampicillin/carbenicillin resistance and were expressed in E. coli AD202 cells.

2.3 Substrates

Substrates involved with NeuB catalysis: PEP, ManNAc, NeuNAc, and UDP-GlcNAc, were purchased from Sigma-Aldrich. Reduced ManNAc (N-acetylmannosaminitol) and reduced NeuNAc (N-acetyl neuraminic borate) was synthesized by Dave Simard at the laboratory of Dr. Martin E. Tanner (see 2.10)

2.4 Over-expression and purification of untagged NeuB construct

An overnight seed culture was grown in 10mL LB supplemented with 100μg/L ampicillin), washed and used to inoculate 2L of LB, which was grown at 37°C. Overexpression of NeuB was induced through the addition of 0.5 mM IPTG at an optical density at 600nm (OD600) of approximately 0.60, with overnight growth (~16 hours) at 20°C. Cells were harvested through centrifugation at 5000 x g for 15 minutes, resuspended in 20 mM Tris HCl (pH 8.5), 150 mM NaCl, and lysed with three passes
through a French press. All purification steps were performed at 4°C. The lysate was centrifuged at 40,000 x g for 35 minutes, passed through a 0.45 μm filter, and loaded directly onto a 20 mL bed volume Q-sepharose FF column (Pharmacia) equilibrated with 20 mM Tris HCl (pH 8.5), 100 mM NaCl. The column was initially washed with 2x column volume of 20 mM Tris HCl, 100 mM NaCl, then eluted with 1.5x column volume gradient of 100 mM NaCl to 1 M NaCl in 20 mM Tris HCl, pH 8.5 at 1 mL/min. Peak fractions containing active enzyme, as assed by SDS-PAGE (Fig.2.4.1a), were then pooled, and ammonium sulfate was added to a final concentration of 1M and adjusted to a pH of 7.0. The protein solution was then loaded on to a 100 ml bed volume phenyl-Sepharose column (Pharmacia) equilibrated with 50 mM sodium phosphate (pH 7.0), 1M ammonium sulfate, and eluted with a 1x column volume gradient from 50 mM sodium phosphate (pH 7.0), 1M ammonium sulfate to 50 mM sodium phosphate (pH 7.0) at 2 mL/min. Peak fractions containing NeuB, as assessed by SDS-PAGE (Fig.2.4.1b), were then pooled and dialyzed overnight against 20 mM Tris HCl (pH 8.5), 100 mM NaCl buffer. The dialyzed protein solution was pressure concentrated by ultrafiltration via the stir cell method to a volume of 5mL and purified in 1 mL injections by Mono-Q anion exchanger (Pharmacia) using a linear gradient of 100 mM NaCl to 1 M NaCl in 20 mM Tris-HCl buffer (pH 8.5). Samples containing eluted protein (Fig.2.4.1c) were then pooled and further dialyzed overnight in 20 mM Tris-HCl (pH 8.5), 100 mM NaCl buffer, concentrated by ultrafiltration to a volume of 2 mL, and applied in 500 μl injections to a Superdex-200 gel filtration column (Pharmacia). Purified protein was pooled
Fig. 2.4.1: SDS-PAGE of the purification of native NeuB. The first column on each gel contains molecular weight standards. a) Fractions off Q-sepharose FF column. b) Fractions off Phenyl sepharose FF column. c) Fractions off Mono Q ion-exchange column. d) Fractions off Superdex 200 gel-filtration column.
(Fig.2.4.1d) and concentrated to $\sim 30$ mg ml$^{-1}$ as measured by the Bradford assay. The protein was assessed to be $>90\%$ pure by SDS-PAGE.

2.5 Over-expression and purification of selenomethionine-substituted NeuB

Native NeuB constructs were transformed into the standard $E. coli$ BL21 (DE3) expression strain for production of selenomethionine (SeMet)-substituted protein. An overnight seed culture grown in 100 mL of LB with ampicillin was washed and used to inoculate 2 L of M9 medium (Sigma) supplemented with 2 mM MgSO$_4$, 0.1 mM CaCl$_2$, 10 mM FeCl$_3$, 1 mg/mL thiamine, and 1% glucose. The culture was then grown at 37°C to an OD$_{600}$ of $\sim 0.6$, at which point 200 mg each of Lys, Thr, and Phe, and 100 mg each of Leu, Ile, Val, Pro and SeMet were added to suppress methionine biosynthesis. After incubation of the culture for a further 15 minutes in order to allow the remaining methionine to be depleted, induction was initiated through the addition of 0.1 mM IPTG (Doublie, 1997). The culture was then grown at 20°C for overnight expression of soluble protein. SeMet substituted protein was then purified as per native NeuB protein, described in section 2.4.

2.6 Over-expression, purification and activity verification of His-tagged enzyme

Preparation of the his-tagged NeuB construct was done by Dave Simard in the laboratory of Dr. Martin E. Tanner in the Department of Chemistry. The $neuB$ gene was cloned into a pET-30 Xa/LIC vector using the Xa/LIC cloning kit protocol (Novagen) and transformed into $E. coli$ strain JM109 (DE3) expression cells. Transformed cells were then grown at 37°C in 500 mL of LB media containing 30 $\mu$g/mL kanamycin. Over-expression of His-tagged NeuB was induced through the addition of 0.5 mM isopropyl-$\beta$-D-thiogalactopyranoside at an OD$_{500}$ of approximately 0.60, and grown for
an additional four hours. Cells were harvested through centrifugation, resuspended in 30 mM triethanolamine-HCl buffer pH 7.5 (containing 10% glycerol, 1 µg/L aprotinin and pepstatin, 1 mM PMSF), and lysed with three passes through a French press. The lysate was loaded directly onto a chelating sepharose nickel affinity column (10 mL, pH 8.0, Pharmacia) was and initially washed with 10 mM and 50 mM concentrations of imidazole. Active protein was then eluted through a linear gradient from 50 mM imidazole to 500 mM imidazole. Fractions containing the enzyme, as determined through SDS-PAGE analysis, were dialyzed overnight in 30 mM triethanolamine-HCl buffer pH 7.5 with 1 mM DTT and 10% glycerol and then flash frozen. \(^{1}\)H- and \(^{31}\)P-NMR spectroscopy was used to monitor the purified His-tagged enzymatic reaction and confirmed that the reaction products were NeuNAc and phosphate, respectively. The unmodified enzyme and the His-tagged enzyme showed similar activities indicating that the presence of the N-terminal modification did not dramatically affect the reaction rate. No reaction could be observed in the presence of 5 mM EDTA, confirming that the divalent cation was vital for activity.

2.7 Protein manipulation and storage

All purification steps were performed at 4°C and concentrated by ultrafiltration using Ultrafree-15 centrifugal concentrators (Millipore Corporation) with molecular mass cutoffs below the molecular mass of the protein. Concentrated protein was then stored in 50 µl aliquots, plunged rapidly into liquid nitrogen and stored at -80°C.

2.8 Protein mass analysis

Mass spectrometry analysis was performed in order to determine the presence of SeMet substituted protein. All mass spectrometry analysis were performed by Dr.
Shouming He (in the laboratory of Dr. Stephen Withers) on a PE-Sciex API 300 quadrupole mass spectrometer interfaced to a reverse phase column. Samples of 100 µg or greater were submitted for analysis. Native NeuB was observed to have a molecular mass of 38,370 Da (theoretical mass of 38,347 Da) and SeMet substituted NeuB was observed to have a mass of 38,846 Da (expected mass of 38,840 Da), thereby indicating full SeMet substitution.

2.9 Static Light Scattering

Static light scattering experiments were kindly performed by Richard Pfuetzner (in the laboratory of Dr. Natalie Strynadka) at 25°C on a Superdex 200 gel filtration column using 20 mM Tris-HCl (pH 8.5), 100 mM NaCl. Refractive index and Mini-dawn light scattering detectors (Wyatt Technology) were calibrated using BSA (Sigma). Native NeuB samples were observed to have a molecular mass of 77,780 Da, thereby indicating the presence of a dimer in solution (theoretical monomeric mass of 38,347 Da).

2.10 Reduction of N-acetyl mannosamine and N-acetyl neuraminic acid

Experimental procedures pertaining to the reduction of substrates and products were done by Dave Simard in the laboratory of Dr. Martin Tanner. Sodium borohydride (0.08 g, 2.0 mmol) was added to a solution of N-acetylmannosamine (ManNAc) (0.21 g, 1.0 mmol) in water (10 mL) and the mixture was stirred at room temperature for 2 h. The solution was neutralized using Amberlite IRP-64 ion exchange resin, filtered and lyophilized. The white solid was refluxed in methanol (50 mL) for 30 min, and then concentrated under reduced pressure. The oily film was dissolved in water and lyophilized to give a white solid (0.16g). Reduction of N-acetyl neuraminic acid was done in a similar manner.
2.11 Crystallization

All crystallization trials were performed using the hanging drop vapor diffusion method (McPherson, 1990) and 24-well crystallization plates (Hampton Research). Purified NeuB at a concentration of 15 mg ml\(^{-1}\) and in the presence of 5mM MnCl\(_2\) and a variety of substrates (10 mM ManNAc/reduced ManNAc, PEP/NeuNAc/reduced NeuNAc) were used for all initial screens. A variety of commercial sparse matrix screens (Jancarik and Kim, 1991), such as Crystal Screens I & II, Index Screen, PEG/Ion Screen, etc. (Hampton Research), were used to determine potential crystallization conditions. Screens were set up in the following manner: equal volumes of concentrated protein solution and crystallization solution were pipetted and mixed on to a plastic cover slide. The cover slide was then sealed over a well containing 0.5 mL of respective crystallization solution and allowed to equilibrate over time. Additional two dimensional grid screens were performed, in which the precipitant concentration and pH were varied in order to determine a suitable condition to promote crystallization of the protein. All crystallization solutions were filter sterilized with 0.2 \(\mu\)m filters prior to their use.

The initial condition resulting in the formation of small NeuB crystals, 2.1 M DL-Malic Acid (pH 7.0), was determined using Index Screen (Hampton). In order to produce larger crystals, precipitant concentration and pH were refined through several grid screens, leading to large crystals that diffracted to high resolution on the home source (Fig.2.11.1). Orthorhombic crystals (0.4-0.5 mm) in the space group \(P2_12_12\) were obtained using 1.75 M Malic acid (pH 6.2) as the precipitant. The largest crystals were formed using 2 \(\mu\)l of a 15 mg/mL protein solution with 1 \(\mu\)l of well solution and growth
taking place at 18 °C. Initial crystals appeared 24-48 hrs, with max growth taking place within 1 week.

![Image of crystal](image)

**Fig. 2.11.1:** Crystals of NeuB. The longest dimension of the hanging drop diffusion produced crystal was approximately 0.5 mm.

Due to the use of high concentrations of malic acid as the precipitant for crystallization, the NeuB active site contained bound malate coordinating the metal cofactor (malate having some chemical similarity to PEP). In order to remove the malate from the active site, native NeuB crystals were soaked in 2 M sodium phosphate, pH 6.2, 5 mM MnCl₂, 10 mM substrates or product (PEP/rManNAc or rNeuNAc) for 24 hrs in order to facilitate buffer exchange. There was no visual difference in crystals upon transfer to an alternate buffer.

### 2.12 Preparation of crystals for X-ray diffraction analysis

In order to protect crystals from radiation damage during data collection, crystals were soaked in 20% (v/v) ethylene glycol (acting as the cryo-protectant), 2 M sodium phosphate (pH 6.2), 10 mM substrate/product and 5 mM metal for 5 minutes then
mounted on a cryo-loop. Loops were then mounted on the goniometer with a cryostream from an Oxford Cryosystems cryocooler maintaining the temperature at 100 K.

Protein crystals were transferred to the synchrotron via a Taylor-Wharton CP100 dry dewar that was pre-cooled with liquid nitrogen the previous night. Diffraction quality of crystals was analyzed prior to their storage in the dewar. Crystals that were determined to be suitable for data collection were transferred to liquid nitrogen using pre-cooled cryo tongs. Plastic vials were submerged in liquid nitrogen and crystals were sealed into frozen vials and stored on metal canes in the dewar.

2.13 Data collection and processing

Data collection on the home source utilized a MAR345 image plate detector (MAR Research) on a Rigaku RU-200 rotating anode X-ray generator (50 kV, 100 mA) with Osmic focusing optics. Data sets were collected by exposing the crystal to an x-ray beam and rotating the spindle axis over a set oscillation range. Typically, an oscillation range of 0.5 or 1.0° was used for data collection. Crystals were exposed to x-rays over a set amount of time based on maximizing diffraction intensity while minimizing crystal decay. Following exposure, the detector was scanned and an electronic image of the diffraction pattern was stored and analyzed.

High resolution and anomalous diffraction data were collected at the Advanced Light Source (ALS) in Berkeley, CA using beamlines 8.2.1 and 8.2.2. Diffraction data was collected using an ADSC Quantum Q210 CCD detector at beamline 8.2.1, and an ADSC Quantum Q315 CCD detector at beamline 8.2.2. For single-wavelength (SAD) and multi-wavelength anomalous diffraction (MAD) data collection, an X-ray fluorescence scan was first measured for a SeMet-substituted crystal. This scan was done
in order to determine the peak wavelength that would maximize \( f' \) and the inflection wavelength that would minimize \( f' \) (Hendrickson, 1991). Highly redundant SAD data sets were collected at the peak wavelength only. MAD data sets were collected at the peak, inflection and remote wavelength, in that specific order. Initial values for \( f' \) and \( f' \) at the peak and inflection wavelength were determined from X-ray fluorescence spectra using the program CHOOCH (Evans and Pettifer, 2001), whereas theoretical values for the remote wavelength were used (Sasaki, 1989).

Native data reduction and processing was done using DENZO and SCALEPACK (Otwinoski and Minor, 1997), and NeuB complex data was processed using MOSFLM (Leslie, 1992) and the CCP4 program suite (Potterton et al., 2003). Initial unit cell dimensions and crystal orientations were determined using the autoindexing function of MOSFLM (Leslie, 1992). Data collection parameters such as optimal starting oscillation angle, number of diffraction images, and optimal oscillation range were determined using the "strategy" function of MOSFLM (Leslie, 1992). Data processed through DENZO (Otwinoski and Minor, 1997) first involved collecting all diffraction images and reducing them to a set of indexed reflections and their integrated spot intensities. From this reduced data, SCALEPACK (Otwinoski and Minor, 1997) was used to combine all image intensities on a common scale through application of a scale factor and a \( B \)-factor. Post refinement of cell parameters was carried out in the initial step of data processed through MOSFLM (Leslie, 1992). Two segments, 45 degrees apart, of the crystal were used for post-refinement in order to minimize differences due to crystal slippage during data collection. Following refinement, all images were integrated together to create a set of indexed reflections. These reflections were then scaled and merged using the program
SCALA, found in the CCP4 software suite (Potterton et al., 2003). Friedel mates from anomalous scattering data were not merged during the processing stages. Intensity parameters from all datasets were then converted to structure factor amplitudes and further processed through programs found in the CCP4 software suite. Two important parameters, the number of molecules per unit cell ($n$) and the solvent content of the unit cell ($V_{\text{solv}}$) were determined at this stage. The equation $V_M=\text{(unit cell volume)}/[n*(\text{mw of protein})]$, was used to solve $n$, where $V_M=1.68-3.53\text{ Å}^3/\text{dalton}$ (Matthews, 1968), whereas $V_{\text{solv}}$ was approximated through the equation $V_{\text{solv}}=1-1.23/V_M$, where it is assumed that most proteins have a partial specific volume of 0.74 cm$^3$/g.

2.14 Structure Determination and Refinement

The structure of NeuB was determined through the collection of anomalous diffraction of a highly redundant single wavelength (SAD) data set from orthorhombic crystals grown using selenomethionyl incorporated protein. Anomalous data was collected at the SeMet peak wavelength of 0.9796 Å to a resolution of 2.5 Å. A total of eight out of a possible ten Se atom sites were identified using Shake-and-Bake (SnB v2.2) (Weeks and Miller, 1999), with additional site refinement carried out using SOLVE (Terwilliger and Berendzen, 1999). These identified sites were used for further refinement and phasing through the program SHARP (La Fortelle et al., 1997) using the SAD SeMet data set. Electron density maps were improved via solvent flattening using RESOLVE (Terwilliger, 2000), resulting in density maps with an overall figure of merit of 0.65. The initial model was built manually using Xfit from the Xtalview software suite (McRee, 1999). An initial Ca trace was made, based on the appearance of the calculated electron density map, using the Baton method where the distance between linking Ca...
atoms were constrained at 3.8 Å. From the Cα trace, a best fit poly-alanine trace was created by replacing pentamers that closely matched a protein structure library. Side chains were then automatically added using the known protein sequence giving the complete un-refined structure.

The complete structure was refined with CNS v1.1 (Brunger et al., 1998) in order to minimize the R-factor (defined as \( R_{\text{cryst}} = \frac{\sum |F_o - F_c|}{\sum F_o} \)), where \( F_o \) and \( F_c \) are observed and calculated structure factors) while conforming to a set of known geometric restraints (Engh and Huber, 1991). In order to calculate the free R-factor, \( R_{\text{free}} \) (Brunger, 1993) a small subset of the data (10%) was designated for exclusion from refinement. The initial refinement of the manually built model involved 20 steps of rigid body refinement to correct the overall position and placement of the molecules in the asymmetric unit. This model was then subjected to a cycle of refinement involving the following steps. Simulated annealing was carried out over a temperature gradient (2500 to 300 K) followed by 100 steps of positional refinement in order to maximize torsion molecular dynamics. This step was followed by positional refinement of the protein residues through minimization over 200 steps. Under this scenario the arrangement of the atoms in the molecule was refined. Following minimization, an overall B-factor refinement was carried out. Subsequent refinement steps involved minimization, followed by individual B-factor refinement where the weight of the B-factor restriction was gradually adjusted to minimize Rfree, and finally, water picking was carried out to determine the location of water molecules within the structure. The final model was obtained through several rounds of refinement to a resolution of 1.9 Å. Bound malate, rManNAc/PEP, and NeuNAcB were modeled at full occupancy, as was the Mn\(^{2+}\) cofactor.
Parameter and topology files (containing geometric constraint information) required for CNS refinement were obtained for PEP and malate through the HIC-Up database (Kleywegt and Jones, 1998) at Uppsala University. Parameter and topology files for NeuNAcB were obtained through the program XPLO2D (Kleywegt, 1995), whereas respective files for rManNAc were obtained through the Dundee PRODRG2 server (Schuttelkopf and van Aalten, 2004).

Following each cycle of refinement the structure obtained was verified through generation of sigma A weighted $2F_o - F_c$ and $F_o - F_c$ electron density maps (Read, 1986) calculated through CNS and displayed with Xfit. Incorrectly placed residues were manually adjusted based on the $2F_o - F_c$ and $F_o - F_c$ electron density maps and subjected to additional rounds of refinement until the R-factor and free R-factor were minimized.

### 2.15 Structure Analysis

Secondary structure elements for the solved protein model were determined with the program STRIDE (Frishman and Argos, 1995), and all required adjustments were done through visual inspection of output files. Verification of the quality of the models was calculated by PROCHECK (Laskowski et al., 1993). Ramachandran plots (Ramakrishnan and Ramachandran, 1965) were also determined by PROCHECK. Comparisons between the structure of NeuB and various other proteins were done through the programs ALIGN (Cohen, 1997) and with the LSQFit option in Xfit (McRee, 1999). Superposition of the Cα atoms was carried out by minimizing the root mean square deviations (rmsd) between equivalent atoms, as determined automatically in ALIGN, and manually selected in LSQFit. Protein structure figures were created with MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merritt and Bacon, 1997). Figures
consisting of electron density maps were created with Xfit and RASTER3D. Molecular surface maps were created with GRASP (Nicholls et al., 1993). Additional figures were created with PyMOL (DeLano Scientific).

2.16 Coupled Assay

The coupled assay was performed by Dave Simard in the laboratory of Dr. Martin E. Tanner in the Department of Chemistry. A cuvette containing Tris-acetic acid pH 8.3 (150 mM, 0.8 mL final volume), ManNAc (variable), NADH (160 µM), LDH (250 U), NeuNAc lyase (20 U, Jülich Fine Chemicals, Germany), MnCl₂ (1 mM), and DTT (1 mM) was incubated for 10 minutes at 37 °C. Following the initial incubation, PEP (variable) was added and the observed slow background rate of PEP hydrolysis was determined by the decrease in absorbance at 340 nm. The enzymatic reaction was initiated by the addition of His-tagged NeuB (1 µg) and the enzymatic rate was calculated from the observed rate less the background rate. The $K_M$ value for ManNAc was measured in the presence of 1 mM PEP and that for PEP was measured in the presence of 30 mM ManNAc.

2.17 $^{18}$O-Labelling experiment

[2-$^{18}$O]-PEP disodium salt was prepared using slight modifications to previously reported procedures (Bondinell et al., 1971; Vialletelle et al., 1992). The resulting material was 66% enriched in $^{18}$O-isotope at the C-2 position as determined by both $^{31}$P NMR spectroscopy and mass spectrometry. The time “0” reaction mixture contained [2-$^{18}$O]-PEP (5 mg), ManNAc (2.5 mM), DTT (1 mM), MgCl₂ (1 mM), triethanolamine-HCl pH 7.5 (final concentration of 12 mM in a total volume of 600 µL), and D₂O (200 µL). A proton decoupled $^{31}$P NMR spectrum was obtained with the sweep bandwidth
limited to 20 ppm. The synthase (0.3 mg) was added and the reaction was monitored for two hours with spectra taken every 30 minutes.
Chapter 3: RESULTS

3.1 Data Collection and Processing

High resolution native NeuB (malate-bound) and SeMet NeuB crystals were collected as described in the materials and methods section (2.13). Crystals were cryoprotected as described in section 2.12. Based on systematic absences, it was determined that the crystals belonged to the space group \( P2_12_12_1 \), with unit cell dimensions (native) of \( a = 58.8 \), \( b = 76.2 \), \( c = 77.5 \) Å and one molecule per asymmetric unit. The complexed NeuB /Mn\(^{2+}\)/reduced ManNA/PEP crystal was determined to have unit cell dimensions \( a = 58.5 \), \( b = 75.63 \), \( c = 77.9 \) Å. Whereas the complexed NeuB/NeuNAcB crystal was determined to have unit cell dimensions \( a = 58.8 \), \( b = 75.9 \), \( c = 77.5 \) Å. Data statistics for native, SeMet, and substrate complexed NeuB are shown in Table 3.1.1

<table>
<thead>
<tr>
<th>Table 3.1.1: NeuB data collection statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data Collection</td>
</tr>
<tr>
<td>Space Group</td>
</tr>
<tr>
<td>Beamline</td>
</tr>
<tr>
<td>Resolution (Å)</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
</tr>
<tr>
<td>Total Reflections</td>
</tr>
<tr>
<td>Unique Reflections</td>
</tr>
<tr>
<td>Redundancy</td>
</tr>
<tr>
<td>Completeness (%)</td>
</tr>
<tr>
<td>(&lt;I/I_o&gt;)^a</td>
</tr>
<tr>
<td>(R_{sym})^a,b</td>
</tr>
</tbody>
</table>

\(^a\)Values in parentheses refer to the highest resolution bin.
\(^b\)\(R_{sym} = \Sigma (I_{hkl}) - <I>/\Sigma (I_{hkl}) \) where \( I_{hkl} \) is the integrated intensity of a given reflection.
3.2 Structure Determination and Refinement

The structure of the native and complex form of NeuB was determined as described in section 2.14 in the materials and methods chapter of this thesis. Refinement statistics for all structures are show in Table 3.2.1.

Table 3.2.1: NeuB structure refinement statistics

<table>
<thead>
<tr>
<th>Refinement Statistics</th>
<th>Malate complex</th>
<th>PEP/rManNAc complex</th>
<th>NeuNAcB Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>$P2_12_12$</td>
<td>$P2_12_12$</td>
<td>$P2_12_12$</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>30-1.9</td>
<td>30-2.2</td>
<td>30-2.2</td>
</tr>
<tr>
<td>Number of atoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>2683</td>
<td>2683</td>
<td>2683</td>
</tr>
<tr>
<td>Substrate</td>
<td>9</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Water</td>
<td>182</td>
<td>255</td>
<td>156</td>
</tr>
<tr>
<td>$R_{\text{cryst}}$ (%)$^a$</td>
<td>0.2070</td>
<td>0.1863</td>
<td>0.2073</td>
</tr>
<tr>
<td>$R_{\text{free}}$ (%)$^a$</td>
<td>0.2376</td>
<td>0.2481</td>
<td>0.2654</td>
</tr>
<tr>
<td>R.m.s. deviations Bonds (Å)</td>
<td>0.0051</td>
<td>0.0057</td>
<td>0.0063</td>
</tr>
<tr>
<td>Angles (°)</td>
<td>1.30</td>
<td>1.31</td>
<td>1.35</td>
</tr>
<tr>
<td>Average B-factor (Å$^2$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>25.0</td>
<td>15.9</td>
<td>27.7</td>
</tr>
<tr>
<td>Substrate</td>
<td>31.4 (malate)</td>
<td>16.2 (PEP)</td>
<td>43.8 (NeuNAcB)</td>
</tr>
<tr>
<td></td>
<td>25.1(rManNAc)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>30.1</td>
<td>21.4</td>
<td>29.0</td>
</tr>
</tbody>
</table>

Ramachandran plot
(% residues in region):
| Most favourable | 93.7 | 92.4 | 93.0 |
| Allowed         | 6.3  | 7.6  | 7.0  |
| Generously allowed | 0   | 0    | 0    |
| Disallowed      | 0    | 0    | 0    |

$^aR_{\text{cryst}}=(\Sigma|F_o - F_c|)/ (\Sigma F_o)$, where $F_o$ and $F_c$ are observed and calculated structure factors.
Chapter 4: DISCUSSION

4.1 Structural Analysis of PEP and N-acetylmannosaminot-bound NeuB

4.1.1 Subunit Architecture

Each monomer of *N. meningitidis* NeuB consists of 349 residues, and can be divided into two distinct domains that are joined by an extended linker region (Figure 4.1.1). The first domain, consisting of residues 1 to 273, has a fold typical of a TIM barrel (Banner et al., 1975): an eight stranded parallel β-barrel enclosed by eight helices (H1-H8). Two additional small α-helices are present between H2 and H3 of the (β/α)_8 barrel and do not lie in the same plane as the other eight helices. These helices are part of an extended loop region of NeuB consisting of residues 61 to 87. Despite low sequence identity (< 10%), superposition of the secondary structure elements of the first 273 residues of NeuB with other PEP utilizing enzymes such as DAH7PS (see 4.4.1) supports the hypothesis that the enzymes are comprised of a very similar overall fold (rmsd value of 2.1 Å on 273 C-α residues).

The second domain of sialic acid synthase consists of 73 residues from the C-terminal end of the enzyme, with a linker region consisting of residues 274 to 284 joining it to the N-terminal TIM barrel domain. Residues 285 to 349 form an antifreeze-like domain, as seen in a variety of type III AFPs (Davies and Sykes, 1997; Jia et al., 1996). This compact, “pretzel-shaped” fold consists of two identical motifs comprised of four short β-strands and a single 3_10 helix. The two motifs are arranged in a pseudo-dyad symmetry, with the eight total strands forming three small antiparallel β-sheets. Two of the sets of β-sheets are three-stranded, and the remaining set has two strands. An eleven residue loop, containing a two-turn 3_10 helix connects the two motifs together.
Fig. 4.1.1.1: Monomeric structure of NeuB. The TIM barrel and antifreeze-like domain of NeuB are shown. Secondary structure elements are represented as ribbons. Helices that are part of the $\beta/\alpha_8$ barrel are labeled for the monomer and are represented in blue, whereas strands are represented in green. Reduced ManNAc and PEP within the active site of NeuB are shown in stick representation with carbon atoms yellow, oxygen red, nitrogen blue, phosphorus green and the metal co-factor Mn$^{2+}$ magenta. The N terminus, C terminus, and individual helices that are part of the TIM barrel are labeled.
Superposition of the last 64 residues from the C-terminal end of NeuB with other type III AFPs (65 residues) yields a very highly correlated structural alignment (rmsd of 1.0 Å on 64 C-α residues, see section 4.4.2) with the major difference being in the N-terminal region (residues 285-290) of the antifreeze-like domain of NeuB. In NeuB, this region contains an additional alpha helix, part of the extension of the linker region between the antifreeze domain and the TIM barrel domain, which is not present in other type III AFPs.

4.1.2 Oligomerization State

Previous circular dichroism and MALDI-TOF spectrometry studies of *E. coli* NeuB indicated that the enzyme existed as a tetramer (Hwang et al., 2002). It should also be noted that both KDO8PS and DAH7PS exist as homotetramers. However, based on the results of our study, it appears as though *N. meningitidis* NeuB exists as a domain-swapped dimer (Fig. 4.1.2.1) and not a tetramer. Our static light scattering experiments of purified NeuB resulted in the elution of a 78000 Da peak (data not shown), suggesting the presence of a physiological dimer (monomer size is 38347 Da). The x-ray structure reveals two antiparallel monomeric NeuB polypeptide chains bound together in a “jigsaw puzzle” like relationship, with the linker region between the two domains of a single NeuB chain providing a groove to which the TIM Barrel of the opposite NeuB chain sits upon. This binding allows for three major areas of interactions to take place between the NeuB homodimer. The first involves the packing of the C-terminal ends (H6, H7, and H8) of two opposing TIM barrels, allowing for extensive Van der Waals (VdW) and hydrophobic interactions between the 2 monomers. The second involves the packing of the linker region (primarily residues 274-284) of one monomer with an opposing TIM
Fig. 4.1.2.1: Oligomerization of NeuB. Arrangement of the NeuB domain-swapped homodimer. One monomer is represented in blue and green, while the other monomer is represented in red and orange. Secondary structure elements are represented as ribbons. Helices that are part of the \((\beta/\alpha)_8\) barrel are labeled for the monomer and are represented in blue and strands in green. Reduced ManNAc and PEP within the active site of NeuB are shown in stick representation with carbon atoms yellow, oxygen red, nitrogens blue, phosphorus green and the metal co-factor \(\text{Mn}^{2+}\) magenta.
barrel (primarily residues 56-61 and 181-201) of the second monomer, again allowing for an extensive interface of VdW interactions. Interestingly, NeuB contains a proline residue at position 284, which lies at the junction between its linker region and the antifreeze-like domain. Earlier studies in other enzyme systems have suggested that the presence of appropriately positioned proline residues in multi-domain proteins can cause a rigid and extended hinge region, keeping the rest of the monomer globular and allowing the protein to oligomerize via domain swapping (Bergdoll et al., 1997). It is tempting to speculate that Pro284 plays such a role in our observed domain-swapped NeuB homodimer.

The third major region of interaction in the NeuB homodimer lies in the packing of the N-terminal region of the antifreeze-like domain of one monomer with the (β/α)8 barrel of the second monomer. Residues 285 to 291 and residues 312 to 319 of the antifreeze-like domain of NeuB lie in between a large cavity surrounded by the looped regions of residues 68 to 79, and residues 184 to 191, in addition to H4 and H5 of the TIM barrel. The closest distance between these two sections of the dimer is between Arg314 of the antifreeze-like domain and Met63 of the opposing TIM barrel, where the two Cα atoms are separated by 3.8 Å. It is important to note when looking at this particular interface that the side chains of three residues from the antifreeze-like domain, Thr285, Phe288, and Arg314, are positioned directly within the depressed cavity of the interacting TIM barrel (Fig. 4.1.2.2).
Fig. 4.1.2.2: Stereoview of the domain interface within the NeuB homodimer. Individual residues (Thr285', Phe288', and Arg314') from the antifreeze-like domain that are positioned within the active site cavity of the TIM barrel are shown in stick representation.

Sialic acid synthases and fish type III AFP have no obvious functional similarities. However, previous studies have shown a very high sequence similarity between the extreme C-terminal end of NeuB and type III AFPs (Baardsnes and Davies, 2001). The structure of NeuB presented here clearly substantiates the claim that these two domains are in fact similar. The sugar substrate binds within the cleft of the TIM barrel of NeuB; however, its stability is likely maintained by residues from the antifreeze like domain (Phe288 and Arg314). AFPs make extensive hydrogen bonds during ice binding (Davies and Sykes, 1997) such that it would not be surprising that these proteins have a propensity to bind sugar molecules. These observations would add NeuB to the growing list of AFP homologues that interact with sugars and polysaccharides (Davies and Sykes, 1997), and is the first reported homologue of the type III AFP.
4.1.3 Malate-bound NeuB Structure

Crystallization of purified native NeuB enzyme resulted in the binding of the precipitant buffer, malic acid, (see methods chapter 2.11) within the active site. This structure was treated as the native composition of NeuB for the purpose of analysis against all substrate bound NeuB compounds. Following refinement of the malate-bound structure, the metal ion Mn$^{2+}$ and malate were clearly visible and fully occupied within the active site (fig. 4.1.3.1).

Fig. 4.1.3.1: Stereoview of observed electron density of malate and Mn$^{2+}$. Observed density of substrates in initial (prior to additional refinement) Fo-Fc map contoured at 1.5$\sigma$ (blue density). Malate is represented with carbon atoms in yellow and oxygen atoms in red. The bound metal co-factor Mn$^{2+}$ is represented in magenta.

Binding of malate to the active site was likely due to the use of a very high concentration (1.7 M) of the precipitant for crystallization, and its structural similarity to PEP (fig. 4.1.3.2). In addition, bound compounds within the active site of the enzyme potentially enhance the crystallization of the protein.

Structural overlap of all backbone carbon atoms of the malate-bound NeuB structure and the rManNAc/PEP-bound NeuB structure resulted in an overall rmsd value of 0.33 Å (data not shown), indicating a near perfect alignment between the two compounds. Observation of the bound malate within the enzyme (fig. 4.1.3.2), indicated
the compound was within the predicted active site and in a position that could plausibly mimic the location of binding of the natural substrates, PEP and ManNAc.

Fig. 4.1.3.2: Stereoview of Malate, Mn^{2+} bound within the active site of NeuB. Malate is represented with carbon atoms in yellow and oxygen atoms in red. Active site residues are also represented in CPK coloring. The manganese ion is represented in magenta.

It appears as though the enzyme did not undergo major conformational changes between the two complexed structures (details regarding the PEP/rManNAc-bound active site are discussed in section 4.1.4). The malate compound binds within the active site so that O1 and O3 of the compound are in close proximity (2.5 Å) to the Mn^{2+} ion, and in conjunction with His215 and His236, form the tetrahedral coordination of the metal. Malate is further coordinated through binding of its hydroxyl groups and various side chain residues (Ser132, Gln55, Asn184, etc) that could also potentially be involved in binding of the natural NeuB substrates.

Although one can comment on the overall structure of NeuB bound to compounds other than substrates and substrate analogues, it is difficult to comment upon the truly "native" form of NeuB, that is the structure with an unbound active site. There is a
possibility of the enzyme requiring a substance to be bound in the active site in order for it to be in a proper conformation for crystallization. It should be noted that during the transfer of NeuB crystals to sodium phosphate buffer, and in the absence of any substrates, sodium phosphate appears to be bound to the active site in a similar position as malate and PEP (data not shown). None the less, it should be interesting to note whether the protein exists as a monomer or a domain swapped dimer in the absence of any compound in the active site, and whether or not any other conformational changes occur upon binding of the substrates.

4.1.4 Active Site of NeuB bound to PEP and reduced ManNAc

In order to confirm the location of the active site of sialic acid synthase, native crystals were soaked with a large excess of reduced ManNAc (rManNAc or N-acetylmannosaminitol) and PEP (see methods). The reduced ManNAc contains a hydroxyl group as opposed to an aldehyde group at C1, thereby prohibiting the cyclization of the sugar and removing the reactive carbonyl group which would cause the reaction to proceed to completion. Under these conditions, electron density for Mn$^{2+}$, rManNAc and PEP was clearly visible and fully occupied within the active site (Figure 4.1.4.1). Binding of both substrates leads to an increased ordering of the enzyme structure, as indicated in the overall decrease in B-values of the protein (table 3.2.1).
Fig. 4.1.4.1: Stereoview of observed electron density of reduced ManNAc, PEP, and Mn$^{2+}$. Observed density of substrates in initial (prior to additional refinement) Fo-Fc map contoured at 1σ (blue density) and 2σ. (magenta density). Reduced ManNAc and PEP are represented with carbon atoms in yellow, nitrogen atoms in blue, oxygen atoms in red and phosphorus atom in green. The bound metal co-factor Mn$^{2+}$ is represented in magenta.

As with other enzymes that assume a TIM barrel topology, the active site of sialic acid synthase is located in a deep depression at the C-terminal end of the barrel. The active site of both KDO8PS and DAH7PS occurs in a cavity at the interface between subunits of the tetramer; however, with NeuB, the active site occurs in a cavity within the TIM barrel of a single monomer, with additional contacts being made with residues from the antifreeze like domain of another monomer. Since the active site is buried deep within the TIM barrel, the antifreeze-like domain essentially seals off the cavity and provides additional residues that come into close contact with the bound substrates. The majority of the residues involved with the active site come from loop regions, and S2 and S4 of the C-terminal end of the TIM barrel, with additional contacts being made by the helix and coiled region of the linker domain, and the extended 11-residue loop of the anti-freeze-like domain.
Earlier studies have shown that NeuB requires a metal co-factor for activity, with Mn\(^{2+}\) having the greatest effect, and Co\(^{2+}\) to a lesser degree (Sundaram et al., 2004). Our results indicate that the metal co-factor is important both structurally and functionally. It was observed that purified NeuB only formed crystals in the presence of a metal co-factor, with the largest crystals forming in the presence of Mn\(^{2+}\). The metal co-factor is coordinated by six different atoms, creating an octahedral geometry within the active site (Fig. 4.1.4.2). Mn\(^{2+}\) is coordinated by the Nɛ2 of both the highly conserved His215 and His236 at a distance of 2.3 Å and 2.4 Å respectively. Two water molecules, W1 and W2, lay 2.2 Å and 2.4 Å away from the metal. The remaining two interactions involve atoms O2P from PEP and O1 from rManNAc, which sit at a distance of 2.1 Å and 2.6 Å respectively, from the metal co-factor. His215, His236, O2P from PEP, and W1 fill the equatorial positions of the coordination, whereas O1 from rManNAc and W2 fill the axial positions. Binding of the hydroxyl group from the sugar substrate suggests that the carbonyl group of non-reduced ManNAc would be in an ideal position to be activated by the metal ion.
The carboxylate and phosphate groups of PEP make several polar interactions with side chains from conserved residues in the active site (Fig. 4.1.4.2). The majority of contacts are made between the *si* face of PEP and the enzyme, with few contacts being made with the *re* face of the substrate. The phosphate group of PEP is tightly anchored between various serine residues and an asparagine residue. O1P of PEP and Ser132 interact at a distance of 2.7 Å, Ser154 and Ser213 hydrogen bond to O3P of PEP at 2.5 Å and 2.4 Å respectively, and Asn184 lies 3.1Å from O1P of PEP. In addition, Atom O1P of PEP and the backbone nitrogen atom of Ser132 also interact at a distance of 3.0 Å. Fewer H-bonds occur between the carboxylate and the bridging phosphate oxygen. One of the carboxylate oxygen atoms, O1, lies at a distance of 3.0 Å from Nζ of Lys129, and 2.9 Å from Nζ of Lys53. Finally, the bridging oxygen atom, O2, of PEP appears to have a weak polar interaction with Nζ of Lys129 (3.0 Å) and it appears to be coordinated with
a water molecule (W1), at a distance of 3.0 Å. The two vinylic carbon atoms of PEP do not make any substantial contacts with any of the residues of the active site as they are coordinated towards the central region of the cavity, thereby making them freely accessible for interactions with the bound sugar substrate.

All of the mechanisms proposed for the PEP-utilizing synthases imply that the reactions proceed via an open chain form of the sugar, and work on both DAH7PS and KDO8PS support this notion (Asojo et al., 2001; Furdui et al., 2004; Howe et al., 2003; Kaustov et al., 2003; Konig et al., 2004; Liang et al., 1998; Shulami et al., 2004; Shumilin et al., 2004; Wang et al., 2001). Structural studies with rManNAc have enabled us to visualize the binding characteristics of the sugar within the active site of NeuB. Several strong hydrogen bonds are formed between the active site residues and the functional groups of rManNAc (Figure 4.3.2). O5 of rManNAc interacts at a distance of 2.8 Å with Oδ1 of Asp247. Two hydrogen bonds are formed between the hydrogen from O3 of rManNAc and Oδ1 of Asp247 and Ne2 of Gln55 (2.7 Å and 2.9 Å respectively). Tyr186 of NeuB forms a hydrogen bond between the hydrogen of its hydroxyl group and the nitrogen from the acetyl group of rManNAc (3.0 Å). The most distinctive observation in bound rManNAc is the numerous hydrogen bonds associated with the antifreeze-like domain of the opposite monomer. Arg314 interacts with the oxygen atom from the acetyl group of rManNAc through water mediated contacts via W3 and W4. In addition, NH1 from Arg314 is at a suitable distance (3.1 Å) to form a weak polar interaction with O7 of rManNAc. O4 also forms a water mediated interaction with Arg314. Further evidence of the importance of the domain swapped dimer in substrate binding is the presence of Phe288 from the antifreeze like domain, which in conjunction
with Phe112 from the TIM barrel forms a relatively hydrophobic pocket in which the acetyl group from rManNAc is located between. The key arginine residue (Arg314) lies in the centre of this hydrophobic pocket between the two aromatic groups. Other PEP utilizing enzymes do not process sugar substrates containing acetyl groups, hence the absence of similar structural interactions in these related proteins. It appears likely that all PEP utilizing enzymes share the same evolutionary background in terms of their protein fold; however, the increasing diversity of reacting substrates potentially influenced the formation of domain swapped dimers within a particular group of these proteins.

The aforementioned polar interactions between the acetyl group of the sugar substrate and the residues of the antifreeze-like domain of the protein suggest that the size of the acyl side chain can play a significant role in enzymatic activity. A recent study by Sundaram et al. concluded that NeuB exhibits similar binding affinity for ManNAc analogs with varying acyl side chains; however, the catalytic activity of the enzyme decreased with increasing acyl side chain length (Sundaram et al., 2004). Based on the limited distance between the PEP and sugar substrate binding site, it appears as though ManNAc structures with longer acyl chains (propanoyl, butanoyl, etc.) that bind at Arg314 between Phe112 and Phe288, would clash with PEP and the metal ion. The C3 atom of PEP and C1 of ManNAc would presumably be at an unfavorable distance due to the presence of bulkier acyl chains in the active site, thus resulting in reduced catalytic activity.

The electrostatic surface of the active site cleft is represented in fig. 4.1.4.3 and shows the majority of the active site cleft is negatively charged, whereas only a small
portion, deep within the cavity, is positively charged. This basic region (arising primarily from the conserved Lys53 and Lys129) enables the tight binding of the negatively charged phosphate and carboxylate groups of PEP, which sits in the deepest portion of the active site cavity. Another, negatively charged depression sits below the si face of the PEP substrate (Fig. 4.1.4.3) and towards the opening of the active site channel, which is the site of sugar binding within this constrained active site. The overall negative charge of the binding pocket would lead to the stabilization of an oxocarbenium ion intermediate. Positioned directly over this area are the side chains from the residues of the antifreeze-like domain, most notably Arg314.

![Molecular surface representation of the active site cleft.](image)

**Fig. 4.1.4.3** Molecular surface representation of the active site cleft. Only the TIM barrel from one monomer is shown, as the antifreeze-like domain from the adjacent monomer has been left off in order to visualize the area within the active site. The surface is color-coded according to the electrostatic potential (red: negative potential, blue: positive potential). Reduced ManNAc and PEP are shown in stick representation in CPK colors. Mn^{2+} is represented in magenta.

Multiple sequence alignments of *N. meningitidis* NeuB with homologs from other species (Fig. 4.1.4.4) support the importance of Arg314 and all other residues associated with PEP and sugar binding as they are conserved across 6 different species. The importance of the conserved Arg314 in the active site is further supported by the conservation of the adjacent Pro313, (likely due to its conformational role in positioning the adjacent Arg314.
side chain correctly in the active site) and a previous study involving the inhibition of *S. agalactiae* NeuB through the addition of an arginine directed modifying reagent, phenylglyoxal (Suryanti et al., 2003). Collectively, the role of Arg314 in the catalysis of sialic acid from NeuB, provides evidence of the structural and mechanistic importance of the formation of the NeuB domain-swapped dimer. Despite the fact that binding of PEP and the sugar molecule occurs in a deep cavity observed within the TIM barrel of one monomer, important protein contacts occur between the acetyl group of ManNAc and the antifreeze-like domain of second monomer. This domain-swapped structure creates a catalytic site from multiple NeuB peptide chains, a structural and mechanistic feature not seen in other PEP utilizing enzymes.
Fig. 4.1.4.4 Multiple sequence alignment of NeuB orthologues from several bacterial species. The *N. meningitidis* NeuB sequence is highlighted in *dark blue* and in *bold face*. The secondary structure of *N. meningitidis* NeuB is shown above the sequence alignments, with *orange lines* representing coils, *red block arrows* representing strands, and *aquamarine cylinders* representing helices. Conserved active site residues of NeuB within bacterial species are represented in *magenta* boxes, whereas other active site residues are highlighted with a *green* box. Conserved active site residues of NeuB from the domain swapped partner are represented by red boxes. Other conserved residues are indicated with a *yellow* box. The numbering of the *N. meningitidis* sequence is represented below the multiple alignment. Sequence alignments were calculated using the program ClustalW (46). Sequences from the following species were included (TrEMBL accession codes in brackets): *Aeromonas caviae* (Q9R9S2), *Pseudomonas aeruginosa* Q8KH52), *Prochlorococcus marinus* (Q7V953), *Neisseria meningitidis* (serogroup B) (Q7DDU0), *Campylobacter jejuni* (gene: NeuBl) (Q7BC41), and *Escherichia coli* (Q46675).
4.2 Structural analysis of N-acetyl neuraminic borate-bound NeuB

Binding of NeuNAcB to the active site of NeuB was done in a similar manner as the rManNAc/PEP-bound complex (see methods chapter 2.11). Similar to the reduced ManNAc compound, the reduced NeuNAc compound contains a hydroxyl at C2 as opposed to a reactive aldehyde group. This prevents the cyclization of the compound and allows one to visualize the binding of the linear form of sialic acid within the active site. However, the reduction of sialic acid resulted in the incorporation of a boron atom (due to the use of sodium borohydride, see methods chapter 2.10) to the linear compound. Incidentally, this compound would represent an oxocarbenium ion intermediate analogue (fig. 1.4.3) and would prove to be useful for analysis of the active site architecture at intermediate steps of the catalytic reaction.

Following refinement of the NeuNAcB-bound structure, NeuNAcB was clearly visible and fully occupied within the active site (fig. 4.2.1).

Fig. 4.2.1: Stereoview of observed electron density of NeuNAcB. Observed density of substrate analogue in initial (prior to additional refinement) Fo-Fc map contoured at 1.5σ (blue density). NeuNAcB is represented with carbon atoms in yellow, nitrogen in blue, oxygen atoms in red, and boron in pink. The bound water molecule is in the position normally attributed to the bound manganese ion.
Structural overlap of all backbone carbon atoms of the NeuNAcB-bound NeuB structure and the rManNAc/PEP-bound NeuB structure resulted in an overall rmsd value of 0.30 Å (data not shown), indicating a near perfect alignment between the two models. The NeuNAcB compound was observed to be bound in a similar position and orientation as that observed for PEP and rManNAc within the active site of NeuB (fig. 4.2.2.2a and 4.2.2.2b). Residues involved with substrate binding under both scenarios are in near identical orientations, as one would expect. The only noticeable difference, in terms of active site residue positioning, between the two structures involves a slight shift in the orientation of Tyr186 and Asn184. Both residues are located in close proximity to the bond formed between C1 of ManNAc and C3 of PEP, hence the possibility that the formation of the bond lead to a slight shift in positioning of both residues. The atoms derived from ManNAc in NeuNAcB are in a similar position as its respective counterpart in the rManNAc/PEP-bound complex. However, the PEP derived atoms (most notably that of C1) of NeuNAcB are shifted closer towards the ManNAc derived atoms, due to the observed bond formed between the two compounds.
Fig. 4.2.2: Binding of NeuNAcB within the active site of NeuB. a) Stereoview of the interactions of NeuNAcB and active site residues of NeuB. NeuNAcB is represented with carbon atoms in yellow, oxygen atoms in red, nitrogen in blue, and boron in pink. Active site residues are represented in CPK coloring. The manganese ion is represented in magenta. b) Stereoview of the structural overlap between active site residues of NeuNAcB-bound NeuB vs. rManNAc/PEP-bound NeuB. Carbon atoms from residues/substrates from the NeuNAcB structure are represented in orange, whereas rManNAc/PEP carbon atoms are represented in cyan. The manganese atom is represented in magenta and water molecules are represented in cyan.

A key difference between the two complexes is the presence of a water molecule in place of the Mn$^{2+}$ co-factor bound to the active site of NeuNAcB. Unsuccessful attempts to bind the metal co-factor were made despite the addition of increasing
concentrations of metal in crystal soaking conditions (5-45 mM). The absence of the metal could potentially be as a result of two factors 1) the presence of a boron atom inhibiting the binding of Mn$^{2+}$ due to its close proximity with the metal ion binding site and 2) removal of Mn$^{2+}$ from the active site due to the formation of sialic acid, thereby negating the need for the metal co-factor. Although the first reason would appear to be the most likely cause of the absence of the metal ion, further studies will be required to make a concrete conclusion.
4.3 Enzymatic Analysis of NeuB

4.3.1 Kinetic Analysis of NeuB

Kinetic analysis of recombinant NeuB was done by Dave Simard in the laboratory of Dr. Martin E. Tanner in the Department of Chemistry. In order to measure the kinetic constants for the NeuB reaction it was useful to develop a continuous coupled assay. Prior enzymatic studies for NeuNAc formation were performed through a stopped thiobarbituric assay (Warren, 1959). The development of a coupled assay that monitors the overall conversion of PEP into pyruvate was made possible through the availability of commercially purified NeuNAc lyase. In the presence of high levels of NeuNAc lyase, any NeuNAc that is formed will be cleaved to pyruvate and ManNAc. Lactate dehydrogenase, which catalyzes the reversible conversion of pyruvate to lactate, is used to detect the break down of pyruvate. The aforementioned reaction requires the consumption of NADH, which can be easily detected due to its strong absorbance at $A_{340}$. Absorption of NAD$^+$ is significantly weaker than that of NADH at 340 nm. This assay was used to obtain the kinetic constants of $k_{cat} = 0.9 \text{ s}^{-1}$, $K_{M,\text{PEP}} = 0.25 \text{ mM}$, and $K_{M,\text{ManNAc}} = 9.4 \text{ mM}$ (150 mM Tris-acetic acid, pH 8.3, 1 mM Mn$^{2+}$). These values agree reasonably well with those previously reported for the wild type enzyme isolated from \textit{N. meningitidis} and assayed under similar conditions ($K_{M,\text{PEP}} = 0.04 \text{ mM}$, and $K_{M,\text{ManNAc}} = 6.3 \text{ mM}$ (150 mM Tris-acetic acid, pH 8.3, 5 mM Mn$^{2+}$)) (Blacklow and Warren, 1962).

4.3.2 Mechanistic Analysis of NeuB

The sialic acid mechanism was analyzed via labeled [2-$^{18}$O]-PEP, prepared with a 66% enrichment of a single $^{18}$O-isotope as determined by mass spectral analysis.
NMR spectroscopy was then used to confirm the location of the isotope at the C2 position of PEP. The substitution of an $^{16}$O-isotope for an $^{18}$O-isotope at a position that is singly bonded to a phosphorus atom will result in an upfield shift in the corresponding $^{31}$P NMR signal (Cohn and Hu, 1978). This effect can clearly be seen in the spectrum of the starting material, which shows a 2:1 ratio (Fig. 4.3.2.1, t=0) of two phosphorus signals ($\Delta$ppm = 0.02) for PEP. Following the addition of NeuB, the signals corresponding to PEP were gradually replaced by those corresponding to inorganic phosphate (Figure 4.3.2.1, t = 60 min). In the absence of ManNAc, no change in the signals was observed, thereby ruling out the possibility that a phosphatase impurity was responsible for this reaction. Examination of the resulting signals also showed a 2:1 ratio of $^{18}$O-labelled to unlabelled compound ($\Delta$ppm = 0.02) confirming that the label was fully retained in the inorganic phosphate and that the reaction took place via a C-O bond cleavage process (Figure 4.3.2.1). The $^{18}$O-label would have been seen as part of the sialic acid product if the reaction had occurred via a P-O bond cleavage process.

Fig. 4.3.2.1: $^{31}$P NMR spectra monitoring the conversion of partially labeled [2-$^{18}$O]-PEP into partially $^{18}$O-labelled phosphate by NeuB. The lower panel shows the spectrum before the addition of NeuB. The upper panel shows the spectrum taken after one hour of incubation.
4.3.3 Proposed Catalytic Mechanism for Sialic Acid Synthase

Several issues pertaining to the understanding of the sialic acid synthase reaction pathway can be addressed based on the structural and mechanistic observations from this particular study. Although the reduced form of ManNAc contains a hydroxyl group instead of an aldehyde at C1, it is presumed that the sugar molecule with the carbonyl group would bind in a similar fashion. At this position, the proximity of the C3 carbon of PEP with C1 of rManNAc is entirely consistent with the observed C-O bond cleavage mechanism in which the initial step involves the formation of a new C-C bond between these groups. The proximity of the rManNAc C1 hydroxyl to the metal ion (2.5 Å) strongly suggests that the addition is promoted via electrostatic catalysis in which the metal polarizes the carbonyl. Under this scenario the \( si \)-side of PEP would nucleophilically attack the \( si \)-side of ManNAc at the C=O double bond, agreeing with the findings by Sundaram et al.\cite{Sundaram}. The intimate interaction of the carbonyl group with the metal ion, and its importance in promoting nucleophilic attack, explains the differences in enzyme activity observed with varying divalent metal ion bound in the active site, with \( Mn^{2+} \) exhibiting the greatest catalytic influence on the mechanism \cite{Sundaram}. This observation differs quite notably from recent reports on the metal-dependent KDO8P synthase and DAH7P synthase that state the metal does not have a direct catalytic role \cite{Furdui, Shulami, Shumilin}. Instead, the metal is proposed to be important in maintaining the correct orientation of substrates/amino acids within the active site such that the delivery of water is facilitated. Recent studies have shown that sialic acid synthase condenses the \( si \) face of PEP with the \( si \) face of the aldehyde group of ManNAc to form sialic acid.
(Sundaram et al., 2004). The 3D structure of the substrate bound NeuB is consistent with this conclusion as PEP is bound in such a position that would facilitate the si face addition of the substrate to the sugar molecule. C1 and C2 of PEP are positioned towards the centre of the cavity where residues from the antifreeze-like domain are positioned, and are twisted (~ 30°) from planarity with the carboxyl group of the substrate, to facilitate the si face condensation. This deviation from planarity is unusual; however, it appears to be critical for the mechanism to proceed via nucleophilic addition of C3 of PEP to the sugar substrate. The twist in PEP is also evident to a lesser degree in PEP bound DAH7P synthase structures (Konig et al., 2004; Shumilin et al., 2003; Shumilin et al., 2004). The greater degree of deviation from planarity in the bound PEP of NeuB is possibly due to the presence of residues from the anti-freeze like domain. Whereas all the active site residues from DAH7PS come from a single domain, NeuB contains active site residues from an alternate domain that is positioned at a slightly different location, requiring for a greater twist in the C1 and C2 atoms of PEP.

PEP-utilizing enzymes that catalyze aldol condensations via a C-O bond cleavage are expected to proceed via the formation of a tetrahedral intermediate (Fig. 1.4.3). This intermediate may be formed in a two step process where the addition of PEP to the aldehyde first generates an oxocarbenium ion intermediate, followed by the addition of water to give the tetrahedral adduct (shown in Fig. 1.4.3). On the other hand, the water may add in parallel to the addition of PEP to give the intermediate in one step. Experiments performed on KDO8P synthase, in which a cationic mimic of the intermediate has been shown to be a potent inhibitor of enzyme activity (Asojo et al., 2001; Kaustov et al., 2003; Wang et al., 2001), has provided the strongest evidence to
date for the oxocarbenium-like transition state. Looking at the active site of NeuB, electrostatic stabilization of this intermediate may come from anionic residues such as Glu25, Glu134, Glu234 (Fig. 4.3.3.1). On the other hand, the highly anionic nature of PEP itself may be enough to provide the required electrostatic stabilization. The formation of the tetrahedral intermediate would then be completed through the addition of water to the C2 position of the adduct. There are two possibilities for this intermediate step, one water molecule, W4, sits 3.3 Å away from the re-face of PEP, and the other, W2, sits 3.9 Å away from the si-face of PEP (Fig. 4.3.3.1). Glu134 is the likely candidate for carrying out the deprotonation of the re-faced water molecule. Alternatively, metal assisted deprotonation of the si-face water molecule could assist in hydroxide delivery to the C2 position of PEP. Glu25 or Glu234 could potentially act as the catalytic base for the deprotonation step of the si-face water molecule. Further analysis would be required to determine the exact nature of the formation of the tetrahedral intermediate.

Fig. 4.3.3.1: Stereoview of proposed catalytic residues involved in the NeuB reaction. H-bonds are indicated as black dotted lines. The covalent bond formed in the condensation reaction between atoms of PEP and ManNAc is indicated with a green dotted line. The metal and rManNAc interaction is indicated with a red dotted line. Catalytic residues and substrates are represented as sticks in CPK colors. Water molecules are cyan spheres and the metal ion is magenta.
The breakdown of the tetrahedral intermediate with loss of phosphate would likely occur in a similar catalytic manner. Ionization of the C2 hydroxyl could be assisted by the involvement of an active site base such as Glu25, Glu134, or Glu234, or by coordination to the metal. The newly created linear form of sialic acid would likely be released into solution where it would spontaneously cyclize to give the pyranose form of the molecule. Alternatively, the enzyme may catalyze the ring opening and ring closing steps of the overall reaction. A mechanistic process involving P-O bond cleavage has been suggested for PEP-utilizing enzymes. This process involves cyclization of the oxocarbenium ion intermediate by attack from the hydroxyl group at C6 (not shown), directly generating the pyranose form of the tetrahedral intermediate. Through a S_N1 process involving a cyclic oxocarbenium ion intermediate, water would then replace the phosphate. The observation of an extended conformation of the bound substrates within the structure rules against such a mechanism. In addition, such a mechanism was ruled out through extensive mechanistic studies involving KDO8P synthase (Liang et al., 1998).
4.4 Comparison of NeuB and Structural Homologous enzymes

4.4.1 Homology between NeuB, KDO8P synthase and DAH7P synthase structures

As previously mentioned in the introduction, NeuB catalyzes a reaction similar to the PEP utilizing enzymes DAH7PS and KDO8PS. Extensive information on the structure of both enzymes bound to PEP and sugar substrate analogues is available (Asojo et al., 2001; Konig et al., 2004; Radaev et al., 2000; Shumilin et al., 2003; Shumilin et al., 2004; Wagner et al., 2000). The most recently solved structure of DAH7PS is a 2.2 Å resolution crystal structure of *T. maritima* DAH7PS in complex with Cd$^{2+}$, PEP and E4P (Shumilin et al., 2004). However, E4P was found to be bound in a non-productive conformation. Both structures are homotetramers with 222 symmetry, and superposition of the monomers from both enzymes yield highly identical structures. Multiple alignments of the primary amino acid sequence of KDO8PS, DAH7PS, and NeuB yield very low sequence similarities between the three enzymes. In addition, of the three PEP utilizing enzymes, NeuB appears to have a requirement for a metal co-factor for catalytic activity (Sundaram et al., 2004), whereas in KDO8PS and DAH7PS it appears as though the metal has no direct involvement with the catalyzed reaction and its mainly required to maintain structural integrity of the active site (Furdui et al., 2004; Shulami et al., 2004).

3D structural alignments between NeuB, and both DAH7PS and KDO8PS indicated a highly similar overall architecture between the three enzymes, with the rms deviation of the superposition being 2.1 Å. The structural alignment between *N. meningitidis* NeuB (core structure colored in magenta) and *T. maritima* DAH7PS (core structure colored in blue) is shown in fig. 4.4.1.1; the alignment with KDO8PS is not shown for reasons of simplicity. Only small structural differences are visible in the
overall alignment of TIM barrels for both enzymes. NeuB consists of an extra helix-loop-helix motif not seen in DAH7PS (yellow in figure), that seals off the active site. This loop presumably acts in conjunction with the active site residues provided by the antifreeze-like domain. An extended coil at the C-terminal end of the barrel of NeuB, acting as an extension of the linker domain that joins the antifreeze-like portion of the enzyme, is also not present in DAH7PS. The N-terminal end of DAH7PS consists of a ferredoxin-like domain (FL) (green in figure) and a linker region (red in figure) connecting the domain to the TIM barrel. No evidence of such a domain is present in NeuB; however, the antifreeze-like domain of the enzyme lies at the C-terminal end of the structure. Another key difference in the additional domains of these structures is the observation that the FL-domain of *T. maritima* does not have a direct role in catalysis (as it is situated away from the active site), but likely plays a role in feedback regulation of the enzyme (Shumilin et al., 2004).
In order to gain a better understanding of the DAH7PS active site, the authors modeled E4P into a productive conformation (Shumilin et al., 2004). The comparison of the binding interactions between the substrates and the active site residues of NeuB and T. maritima DAH7P reveal several similarities and some stark differences, most notably, the shift in bound substrate due to interactions with the antifreeze-like domain (Fig. 4.4.1.2). Overall binding of the PEP substrate within the active site of both enzymes is similar; PEP is anchored near the metal ion deep within the cavity, with C3 of the vinylic carbon pointed towards the centre. The phosphate group of PEP in the NeuB complex makes contact with the metal ion, whereas the phosphate group of the T. maritima
DAH7PS complex is shifted away from the metal ion, so it is not in an ideal position to form significant interactions. Most of the PEP interacting residues of NeuB are negatively charged (due to the prevalence of aspartate and glutamate residues) whereas the PEP interacting residues of DAH7PS are positively charged.

**Fig. 4.4.1.2:** Structural alignment of equivalent active site residues of *N. meningitidis* NeuB and *T. maritima* DAH7PS. Active site residues involved in PEP and sugar binding are represented by stick diagrams for both enzymes. DAH7PS residues and substrates are shown in cyan, whereas NeuB residues and substrates are shown in yellow. Arg314' from NeuB is coloured orange. The metal co-factor Mn$^{2+}$ is represented in magenta for NeuB, and Cd$^{2+}$ is pink for DAH7PS. Nitrogen, blue; oxygen, red; phosphorus, green.

The phosphate group of E4P is anchored between Arg133 and Thr134 of *T. maritima* DAH7PS, with C1 of the sugar directed towards the centre of the active site and at a non-productive distance of 4.2 Å away from C3 of PEP (Fig. 4.4.1.2). When modeled into a productive orientation, the carbonyl oxygen of E4P is positioned a short distance away from the metal ion and bringing the C1 atom of E4P within 2.8 Å of C1 of PEP (Shumilin et al., 2004). ManNAc appears to be only loosely associated with Asn74; however, the lack of a phosphate group potentially explains the lack of tight associations at this end of the sugar molecule. The positioning of the conserved Arg314' and Phe288' in the antifreeze-like domain of NeuB explains the shift in PEP and ManNAc positioning.
relative to the bound substrates in DAH7PS. Key atoms in PEP are positioned towards the two residues of the antifreeze-like domain so that nucleophilic attack of the C3 atom of PEP on the C1 carbonyl of ManNAc can take place. The absence of an acetyl group in E4P eliminates the need for additional contacts to be made with DAH7PS.

Residue Lys131 in *T. maritima* DAH7PS has been implicated as the general acid, whereas Glu164 has been identified as the general base of the reaction (Shumilin et al., 2004). Based on the active site structure of NeuB and analysis of conserved residues from the multiple sequence alignment of NeuB from various organisms, it appears as though Gln55, Tyr186, Asp247, and Arg314' make important contacts with the sugar substrate. In addition, Gln55 also potentially acts as the general acid, and Glu25, Glu134, or Glu234 as the general base. The imidazole ring of His236 possibly serves a dual role in binding the metal co-factor and transferring lone hydrogen electrons during the catalytic process. Site directed mutagenesis of suggested catalytic residues combined with further kinetic and mechanistic studies will be required in order to make more conclusive statements about the production of sialic acid.

### 4.4.2 Homology between NeuB and fish type III antifreeze proteins

Sialic acid synthases and fish type III AFP have no obvious mechanistic similarities. NeuB catalyzes the production of a biologically essential nine carbon sugar molecule, whereas type III AFP decreases the freezing point of water by binding ice crystals and inhibiting their growth. However, previous studies have shown a very high sequence similarity between the extreme C-terminal end of mammalian NeuB and type III AFPs (Baardsnes and Davies, 2001). The 3D structure of NeuB further substantiates the claim that these two proteins are in fact similar in that the extreme C-terminal end of
the protein contains an antifreeze-like domain. Superposition of the 3D structure of the antifreeze like domain of NeuB and type III AFP results in an rmsd value of 1.0 Å on the 64 common residues (Fig. 4.4.2.1). Only a few differences between the two structures exist: the C-terminal end of type III AFP contains an extended coil not seen in NeuB, and the N-terminal end of the antifreeze like domain of NeuB contains a helix that is an extension of the linker region connecting to the TIM barrel of the enzyme. However, the extreme C-terminal end of bacterial sialic acid synthase has a relatively low sequence similarity with its mammalian counterpart and the type III AFPs (Fig. 4.4.2.2), despite the fact that \textit{N. meningitidis} NeuB forms an antifreeze-like domain. Structural analysis of sialic acid synthase from other organisms will be required to further discuss its relationship with the antifreeze proteins.

\textbf{Fig. 4.4.2.1:} 3D secondary structural alignment of the antifreeze-like domain of NeuB and fish type III AFP. Secondary structure elements of both structures are represented by ribbon diagrams with the view being along the pseudo-dyad axis. Differences between the two structures are highlighted, NeuB: orange, core antifreeze-like domain; green, N-terminal helix extension from the linker region connecting to the \((\beta/\alpha)_8\) barrel. Type III AFP: blue, core antifreeze-like domain; magenta, C-terminal coil extension. The root mean square deviation of the alignment of 64 residues was calculated to be 1.0 Å.
There are four known classes of antifreeze proteins (numbered sequentially I-IV) and a single group of antifreeze glycoproteins (Davies and Sykes, 1997). Each of these classes contains proteins that are sequentially and structurally different. Over the past couple of years a growing list of AFP homologues that interact with sugars and polysaccharides has been produced. For example, type I and Ca$^{2+}$ dependent lectins; type IV and Lipoprotein domain (Davies and Sykes, 1997). However, sialic acid synthase would be the first reported case of a structural homologue to the type III antifreeze proteins. Although type III AFPs have no direct role in sugar catalysis, the results of this study suggests that the anti-freeze like domain of NeuB plays a very important role in the binding of the sugar substrate. The sugar substrate binds within the cleft of the TIM barrel of NeuB; however, its stability is maintained by residues from the antifreeze like domain (Thr285 and Arg314). Multiple sequence alignments between the antifreeze-like
domain of NeuB and type III AFPs (fig. 4.4.2.2) do not show the presence of the key arginine residue in AFPs, but it would only seem logical that antifreeze-like proteins would have a propensity to bind sugar molecules due to their ability to form numerous hydrogen bonds in the case of ice binding. In addition, residues involved with ice-binding in the antifreeze proteins do not appear to be conserved in the sialic acid synthases (fig. 4.4.2.2). However, it should be interesting to see if sialic acid synthase has the ability to bind ice crystals (through different contact points), since from a structural standpoint, its flat and mostly neutral surface is similar to that of ice binding proteins.
Chapter 5: Conclusions and Future Directions

5.1 Summary and Significance of Results

It was the overall goal of this thesis to determine the structural and enzymatic characteristics of the enzyme from *N. meningitidis* directly involved in the creation of sialic acid. The importance of understanding the mechanism of sialic acid production is made evident by the vital role this sugar plays in a number of biological processes. Their widespread existence in bacteria enables various pathogens to avert host mammalian host defenses, and makes them an attractive target for the development of novel antibiotics. Despite the fact that both organisms produce sialic acid, the location of the production of the compound differs between mammalian cells and microorganisms, thereby allowing for the development of specific antibacterials that only target pathogenic bacteria. There is also obvious industrial potential for sialic acid synthases in the large scale production of sialic acid containing reagents.

We report the first solved crystal structure of a sialic acid synthase complexed with Mn$^{2+}$ and its natural substrate PEP, and substrate analog rManNAc. In addition, structural analysis of NeuB bound to various other compounds including a non-substrate compound, malate, and an intermediate analogue, NeuNAcB, were discussed and contrasted. It was found that the binding interactions of the active site residues were similar in all three cases, with the absence of a metal co-factor in the NeuNAcB-bound complex being the most significant difference. The absence of the metal co-factor was potentially due to the presence of a boron atom in the NeuNAcB compounds. In collaboration with Dr. Martin Tanner's laboratory, it was demonstrated that the synthase employs a C-O bond cleavage process during catalysis and a new continuous assay to
monitor the reaction kinetics was developed. NeuB exists as a domain-swapped dimer which appears to be mechanistically important for the creation of the NeuB active site, as it provides contacts for enzyme:sugar substrate interactions. More specifically, it appears as though Arg314 from the adjacent monomer plays a contributing role in binding the sugar substrate. In addition, the bound metal appears to play a catalytic role in the production of sialic acid, an observation that contrasts against recent enzymatic research on DAH7PS and KDO8PS (Furdui et al., 2004; Shulami et al., 2004; Shumilin et al., 2004).

5.2 Future Work

Experiments are currently underway to observe the binding interactions of other substrate analogues, inhibitors, and the cyclic form of sialic acid with NeuB. The ability of the cyclic compound to bind within the active site of NeuB should provide some insights as to whether or not the catalyzed compound cyclizes within the active site, or if it becomes cyclized upon its release from the enzyme. Site directed mutagenesis studies of residues involved in the binding of PEP and ManNAc will also further the understanding of the mechanism of sialic acid synthase. Active site residues that are to be manipulated will be determined through the observed 3D structure of NeuB, and should provide clues as to which amino acids act as the specific catalytic acids and bases. As discussed in section 4.3.3, Glu25, Glu134, and Glu234 are thought to play a catalytic role in the enzymatic reaction of NeuB and mutating these specific residues should clarify there roles as catalytic bases and/or as residues involved in the stabilization of the oxocarbenium ion intermediate. Mutating the conserved Arg314 residue should provide insights as to whether the residue is catalytically important, or if it simply plays a
stabilizing role. In addition, other residues found in the antifreeze-like domain that may be of further interest include Phe288, whose importance has been speculated to be related to sugar stabilization. In terms of the entire antifreeze-like domain, it would be interesting to determine whether the extreme C-terminal end of NeuB has a propensity to bind and inhibit ice formation, and whether this activity is similar to that of known antifreeze proteins.

Although this thesis focuses primarily on bacterial sialic acid synthases, future studies would also focus on the structure-function relationships of eukaryotic sialic acid synthases, both the murine and mammalian enzymes. Despite the fact that alignments of sialic acid synthases between eukaryotic and prokaryotic species yield a relatively low sequence identity (~20%) the presence of the antifreeze-like domain in both species suggests that there may be some structural similarities. Structural and mechanistic information derived from this study should assist in the determination of the 3D structures of the eukaryotic enzymes. It should be interesting to note whether the presence of a phosphate group, in the substrate ManNAc-6-phosphate, affects the active site coordination of the mammalian sialic acid synthase. Due to the presence of said phosphate group, and the low sequence identity, it would be presumed that different amino acid residues would be involved in the make up of the active site. Information derived from the mammalian structure would lead to a thorough comparison against the bacterial species and a better understanding of the enzymatic mechanisms driving sialic acid synthesis. The enzymatic contrast between the bacterial and mammalian sialic acid pathways would further contribute to the development of novel antibiotics specifically targeting this mechanism.
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