HEAVY ATOM AND HYDROGEN KINETIC ISOTOPE EFFECT STUDIES ON RECOMBINANT, MAMMALIAN SIALYLTRANSFERASES

By

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by

ERIN E. BURKE
This work is dedicated to my husband, my parents, and to all of my family and friends who have supported me in this endeavor.
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Sialylated glycoproteins and glycolipids are key recognition molecules for a host of biological processes such as cell-cell regulation, cell adhesion, and biological masking. Sialyltransferases are glycosyltransferases that catalyze the biosynthesis of sialylated oligosaccharides in the Golgi apparatus of many prokaryotic and eukaryotic cells. The mechanism of sialyl transfer from activated donor substrate, 5’-cytidine monophosphate N-acetylneuraminic acid (CMP-NeuAc), to terminal positions of carbohydrate groups on glycoproteins and glycolipids is still not fully understood. Kinetic studies on recombinant rat liver α(2→6) sialyltransferase propose a mechanism with a late oxocarbenium ion-like transition-state and general acid catalysis to assist in glycosyl transfer. This dissertation describes the mechanistic study and the transition-state analysis of three recombinant sialyltransferases. The results from this study will provide an increased understanding of the mechanism of glycosyl transfer which may be useful in the future development of new sialyltransferase inhibitors.
The first part of this work describes the synthesis and purification of the isotopically labeled CMP-NeuAc and UMP-NeuAc donor substrates required to conduct the desired kinetic experiments. Details describing a novel enzymatic route for the synthesis of non-bridging phosphate $^{18}$O labeled CMP-NeuAc are also presented in this section. The characterization of these isotopically labeled substrates is shown here as well.

Following the synthesis of the substrates, the next section describes the cloning, overexpression, and purification of three recombinant human $\alpha(2\rightarrow3)$ sialyltransferases, two of which contain either a N-terminal His$_{6\times}$-tag or a C-terminal His$_{6\times}$-tag. The purification yields, specific activities, and kinetic parameters of these recombinant human $\alpha(2\rightarrow3)$ sialyltransferases are also presented.

The dissertation concludes with the discussion of the kinetic isotope effect studies on recombinant human $\alpha(2\rightarrow3)$, rat liver $\alpha(2\rightarrow3)$, and rat liver $\alpha(2\rightarrow6)$ sialyltransferase with the aforementioned isotopically labeled substrates. The kinetic isotope effects that were measured on these enzymes include secondary $\beta$-dideuterium, binding, control, and primary and secondary $^{18}$O leaving group isotope effects. Comparisons were made among isotope effects measured for the recombinant human and rat $\alpha(2\rightarrow3)$ sialyltransferases and for the recombinant rat $\alpha(2\rightarrow6)$ sialyltransferase. The KIE data provide new information regarding the nature of the transition-states for the $\alpha(2\rightarrow3)$ and $\alpha(2\rightarrow6)$ sialyltransferase enzymes.
CHAPTER 1
INTRODUCTION

Sialyltransferases are glycosyltransferases that catalyze the transfer of sialic acid (N-acetylneuraminic acid, NeuAc) from an activated CMP-NeuAc donor substrate to non-reducing termini of glycoproteins and glycolipids with inversion of configuration at the NeuAc glycon. Over the last two decades, research interest in sialyltransferases has increased primarily because these enzymes play a critical role in the regulation of a host of biological processes. The mechanism of sialyl transfer is still not fully understood. Results from kinetic studies conducted previously by Bruner and Horenstein on recombinant rat liver $\alpha(2\rightarrow6)$ sialyltransferase (ST6Gal I) suggested that the mechanism proceeds via a late oxocarbenium ion-like transition-state with general acid catalysis to assist in glycosyl transfer.\(^1\,^2\) The work described in this dissertation represents an investigation into the reaction catalyzed by recombinant $\alpha(2\rightarrow3)$ sialyltransferase from human placenta (h23STGal IV). The enzyme-substrate binding interactions at the phosphate group of the donor substrate are of particular interest. Additionally, comparisons can be made between enzymes in the same family using the information obtained from work completed on recombinant rat liver $\alpha(2\rightarrow3)$ sialyltransferase and from the work previously reported on the recombinant ST6Gal I.

Sialic Acids

In order to understand the significance of studying this class of enzymes, one must first appreciate the functional importance of sialic acids for a host of biological processes. The purpose of this introduction is not to provide a comprehensive report on the subject
of sialic acids, but to merely highlight some of the key roles that sialic acid residues play in these biological processes. This information will emphasize the importance of how studying the enzymes involved in sialic acid regulation could lead to ways in which to control and monitor a wide array of biological pathways.

In nature, sialic acids are linked in the terminal steps of the synthesis of cell surface glycoproteins and glycolipids. The structure of sialic acid is unique in that it contains a highly acidic carboxylate group on the anomeric carbon (Figure 1-1). The negative charge on sialic acids is an important chemical feature of this molecule and it plays a functional role in a variety of biological processes. For example, the negative charge on sialic acids provides this molecule with the ability to attract and repel specific cells and biomolecules. In the case of attraction, the negative charge allows sialic acids to bind to positively charged molecules and assist in their transport.

![Figure 1-1. General structure of free sialic acid and N-acetyl neuraminic acid (NeuAc) which is transferred by sialyltransferases.](image-url)
On the other hand, the population of sialic acid residues on the periphery of cells provides the cell with a net negative charge that is essential for the repulsion of other cells or biomolecules. This is seen in erythrocytes and blood platelets where cell-surface sialic acids can prevent the aggregation of these cells in the bloodstream. Furthermore, the negative charge on these sugars has also been shown to contribute to the viscosity of mucins lining intestinal endothelia cells. Thus, the electrochemical properties of sialic acids appear to influence their unique function in a variety of biological phenomena.

One of the most important roles of sialic acids is their ability to function as recognition elements for key processes. This function is facilitated by their chemical properties and by their location on surface of cells. For example, sialic acids are recognition molecules for bacterial and viral pathogens. The best known example of this was observed over 50 years ago where sialic acid residues were identified as recognition molecules for the binding of influenza A to human erythrocytes and respiratory tract mucins. Since then, researchers have shown that influenza B virus can also bind to sialic acid residues that are N-linked to cell-surface glycoproteins or glycolipids. The binding of influenza to cell surface receptors bearing sialic acids is mediated by the viral protein, Hemagglutinin (HA). HA works in conjunction with viral sialidase (or neuraminidase) during the viral life cycle. Viral sialidase has been suggested to facilitate the spread of influenza virus by cleaving sialic acid residues from the protecting mucin layer of respiratory tract epithelia cells. This research lead to the development of viral sialidase inhibitors such as 2-Deoxy-2,3-dehydro-N-acetyleneuraminic acid (DANA) which are being successfully used today for the treatment of influenza (Figure 1-2).
Figure 1-2. Structure of the viral sialidase inhibitor, DANA.

Another example of sialic acids acting as essential recognition components is in neural development. In this case, polysialic acid, a linear homopolymer of $\alpha(2\rightarrow8)$-linked sialic acids, was discovered attached to the neural cell adhesion molecule (NCAM).\textsuperscript{10} Two polysialyltransferases, ST8Sia II (STX) and ST8Sia IV (PST), regulate the synthesis of polysialic acid on NCAM. Experiments involving NCAM-deficient mice have suggested that polysialic acids on NCAM play critical roles in the regulation of neural cell adhesion, cell migration, neurite outgrowth, and synapse formation.\textsuperscript{11,12} Furthermore, deficiencies and disorganization in polysialic production have been linked to diseases such as schizophrenia and Alzheimer’s disease.\textsuperscript{13,14}

In contrast to their function as recognition molecules, sialic acids are also important for the anti-recognition of certain biomolecules and cells. As the penultimate “capping” molecule for cell-surface oligosaccharides, sialic acids serve as biological masking agents by disguising and shielding their underlying sugars from receptor recognition. Ashwell and Morell documented the first example of this function in 1974. Their experiments investigated the role of sialic acids as masking agents by using sialidase to remove
terminally N-linked sialic acid residues from D-galactose molecules on radiolabeled ceruloplasmin in the bloodstream. Upon removal of these sialic acids, exposed galactosyl residues were quickly recognized by D-galactose receptors and the resulting radiolabeled asialoceruloplasmin disappeared from the bloodstream within minutes. Analysis of the liver showed increased radioactivity, thus signifying the degradation of radiolabeled asialoceruloplasmin.

Another example of the sialic acid masking effect can be seen in the binding recognition of siglecs in the immune system. Siglecs are sialic acid-binding immunoglobulin-like lectins involved in cellular signalling functions and cell-cell interactions in the nervous and immune systems. Siglecs use both cis and trans interactions with sialic acid ligands when binding to the cell-surface. Researchers have shown that the siglec receptor-binding site can be masked by cis interactions with sialic acid ligands. These cis interactions with sialic acids are essential for the regulation of siglec function by preventing or facilitating specific cell-cell interactions when necessary.

Sialic acids have also been implicated in the masking of tumor antigens. In this case, the high sialic acid content on some tumor cells can mask antigen recognition sites. As a result, tumor cells can elude immunological attack and, in some cases, continue to grow uncontrollably. Clinical studies have observed that certain invasive cancer cell lines have hypersialylated cell-surfaces and the patients with these cancers have increased sialyltransferase activities in their blood serum. In response to these results, researchers have been investigating the use of sialidases and sialyltransferase inhibitors as methods for cancer treatment. Studies conducted on chemically induced malignant tumors in
small mammals showed marked tumor regression after treatment with sialidase.\textsuperscript{20}

Additionally, sialyltransferase inhibitors such as KI-8110 have been shown to assist in the reduction of tumor metastases by inhibiting the transfer of sialic acid onto cell-surface oligosaccharides (Figure 1-3).\textsuperscript{21-24} Thus, these findings have opened the door to new possibilities in the development of cancer treatments.

Figure 1-3. Chemical structure of sialyltransferase inhibitor KI-8110.

Since sialic acids participate in such a plethora of biological events, changes in their chemical structure, concentration, or mutations to their biosynthetic pathways can cause severe diseases. Some of the diseases include sialidosis, galactosialidosis, sialuria, and sialic acid storage disorder (SASD).\textsuperscript{25} Sialidosis and galactosialidosis are genetically inherited, lysosomal storage diseases that are characterized by the inability to degrade sialylated glycoproteins due to a deficiency in the production of sialidase.\textsuperscript{26} In contrast, sialuria is characterized by the overproduction of sialic acids in the cytoplasm resulting from a lack of feedback inhibition of the rate-limiting enzyme, uridine diphosphate N-
acetylglucosamine 2-epimerase. Sialic acid storage disorder is the rarest of the aforementioned diseases with less than 150 cases reported worldwide. Most of the reported cases stem from a small region in northeastern Finland. SASD is typified by the accumulation of unbound sialic acid within the lysosomes caused by a defect in sialin, a transmembrane protein responsible for transport of sialic acid out of the lysosome. As a result, patients with SASD have up to 100 times more unbound sialic acid in their urinary excretions than normal. In general, patients suffering with these diseases display neurodevelopmental delays, severe learning difficulties, coarse facial features, spinal abnormalities, skin lesions, and visual impairment.

The functional importance of sialic acids in biological systems is vast. Their unique roles in a variety of cellular events make sialic acids indispensable to life. Therefore, mechanistic studies on the enzymes involved in the biosynthesis and transfer of sialic acids could provide new tools in which to investigate the complex nature of these biomolecules.

**Glycosyltransferases and Glycosidases**

Glycosyltransferases are a class of enzymes that catalyze the transfer of monosaccharide residues from mono- or diphosphate sugar nucleotides to the non-reducing end of extending oligosaccharide chains, or, in general, different aglycon acceptors. These membrane bound enzymes are highly specific for their donor and acceptor substrates, and their function is critical for the proper glycosylation of a myriad of oligosaccharide and polysaccharide chains. In mammalian cells, estimates indicate that well over 100 different glycosyltransferases are required to biosynthesize all known oligosaccharide structures. The resulting glycan chains regulate a diverse range of
cellular functions, including cell-cell interactions and signaling, host-pathogen interactions, neuronal development, embyogenesis, and biological masking.  

Glycosyltransferases are classified by sequence similarity-based families and by the type of mechanism they catalyze, which is retaining or inverting depending on the final anomeric configuration of the product (Figure 1-4). The inverting glycosyltransferase catalyzed mechanism is suggested to follow an S_N2-like reaction whereby a general base deprotonates the incoming nucleophile of the acceptor sugar, thus enabling the direct displacement of the nucleoside diphosphate. In this mechanism, metal ions such as Mg^{2+} or Mn^{2+} are believed to serve as acid catalysts for some glycosyltransferases.

Figure 1-4. Proposed mechanism for inverting and retaining glycosyltransferases and glycosidases from Lairson et al.
The retaining glycosyltransferase catalyzed mechanism is proposed to proceed via a double-displacement reaction with a covalently bound glycosyl-enzyme intermediate. In this mechanism, an aptly positioned amino acid within the active-site functions as a nucleophile to catalyze the reaction.\textsuperscript{30,32} A divalent cation is believed to act as a Lewis acid whereas the leaving diphosphate group has been suggested to serve as a general base by deprotonating the incoming acceptor sugar hydroxyl to activate it for nucleophilic attack. In comparison to inverting glycosyltransferases, retaining glycosyltransferase reactions also proceed through oxocarbenium ion-like transition states. Despite this similarity, the mechanism for retaining glycosyltransferases is still being explored since some of the intermediates have yet to be conclusively identified.

Within the last decade, several crystal structures of glycosyltransferases have been reported.\textsuperscript{28,32,33} Based on recent structural data, glycosyltransferases adopt one of two general folds referred to as GT-A and GT-B (Figure 1-5).\textsuperscript{29} Glycosyltransferases categorized under the GT-A (glycosyltransferase A) fold group typically contain a conserved ‘DXD’ motif, a conical active site cleft formed by two closely associated domains, and \(\alpha/\beta\) proteins with a single Rossmann domain. The ‘DXD’ motif has been shown to play a critical role in metal ion binding and catalysis. The divalent metal cation coordinates the phosphate group oxygens of the sugar nucleotide donor in the enzyme active-site.\textsuperscript{34,35} Additionally, binding of the nucleotide has been mainly observed on the N-terminal domain of GT-A enzymes. Some of the glycosyltransferases that have been classified under the GT-A fold group include phage T4 \(\beta\)-glucosyltransferase, glycogen phosphorylase and \(\alpha\)-1,3-galactosyltransferase.\textsuperscript{29,36}
The GT-B (glycosyltransferase B) fold group consists of two Rossman-like β/α/β domains that are separated by a deep substrate-binding cleft. Nucleotide binding for GT-B enzymes takes place on the C-terminal domain while the acceptor substrates bind to the N-terminal domain. The GT-B superfamily encompasses a diverse group of prokaryotic and eukaryotic enzymes that are responsible for a variety of processes ranging from the production of biologically active antibiotics to cell wall biosynthesis and gene transcription.37

Figure 1-5. 3-D structural representations of the GT-A and GT-B fold groups of glycosyltransferases from Coutinho et al.29

In contrast to glycosyltransferases, glycosidases execute further modifications to glycosylated biomolecules by catalyzing the cleavage of sugar residues. This class of enzymes uses water as a nucleophile to trim carbohydrate residues from these biomolecules in order to meet the requirements for a variety of biological processes.

Glycosidases follow similar mechanistic paths to those described above for glycosyltransferases, but with a few exceptions. The characteristic mechanism for retaining glycosidases involves a pair of aspartic or glutamic acid residues in enzyme active-site, with one functioning as a nucleophile and the other acting as a general acid/base catalyst. Unlike retaining glycosyltransferases, key intermediates in the
retaining glycosidase mechanism such as the covalently bound glycosyl-enzyme intermediate have been conclusively identified and characterized using crystallographic and spectroscopic methods. Recently, the Withers laboratory characterized this covalently bound glycosyl-enzyme intermediate for the hen egg-white lysozyme (HEWL) mechanism.38

**Fucosyltransferases**

Although glycosyltransferases have not been as well characterized as glycosidases, one member of the glycosyltransferases superfamily that has been closely studied is fucosyltransferase. Fucosyltransferases catalyze the transfer of L-fucose from an activated GDP-fucose donor substrate to oligosaccharide chains linked to proteins or lipids. In recent years, the mechanism for α(1→3) fucosyltransferase V (FucTV) has been investigated using kinetic isotope effect experiments and inhibitor studies.39-41 FucTV catalyzes the final step in the biosynthesis of sialyl Lewis X and Lewis X fucoglycoconjugates. These fucoglycoconjugates play an essential role in the regulation of cell-cell interactions for a variety of immune system processes.

Kinetic isotope effect and pH-rate studies conducted on FucTV suggest that the mechanism for fucosyltransfer is base catalyzed where the L-fucose is transferred to acceptor sugars with inversion of configuration.39 Results from secondary isotope effect studies using deuterated GDP-[1-2H]-Fucose as the donor substrate indicated that cleavage of the glycosidic bond occurs prior to nucleophilic attack as illustrated in Figure 1-6.40 Furthermore, the transition-state structure is similar to glycosidases in that it is proposed to have a flattened half-chair conformation with considerable oxocarbenium ion character at the anomeric position.
Product inhibition studies on FucT V have revealed that the mechanism is an ordered, sequential, Bi-Bi mechanism in which the GDP-Fuc binds first followed by the acceptor sugar. FucTV also requires a metal co-factor, typically Mn$^{2+}$, to achieve optimal catalysis. Furthermore, FucTV can use both charged and uncharged sugar acceptor substrates such as N-acetyllactosamine (LacNAc) and sialyl LacNAc, respectively. This sugar acceptor substrate variability is similar for sialyltransferases however, the FucTV donor substrate, GDP-Fuc, does not contain the highly acidic carboxylate group on its anomeric carbon like the sialyltransferase donor substrate, CMP-NeuAc. Thus, this group will undoubtedly alter the enzyme-donor substrate reactivity when compared to GDP-Fuc. Nevertheless, the mechanism for FucTV may provide some insight into the nature of the sialyltransferase and other glycosyltransferase catalyzed reactions.
Sialyltransferases

As a subfamily of glycosyltransferases, sialyltransferases are also localized in the Golgi apparatus and their topology is characteristic of a type II membrane protein with a short cytoplasmic domain, an N-terminal signal anchor and a large luminal catalytic domain (Figure 1-7). There are presently 20 cloned cDNA’s of sialyltransferases isolated from bacteria, insects, and mammals. Their nomenclature and function are determined by the different acceptor sugar substrates that sialyltransferases bind to in the transfer of NeuAc. For example, the recombinant h23STGal IV catalyzes the transfer of NeuAc from CMP-NeuAc donor substrate to the C3 terminal hydroxyl of Galβ1,4GlcNAc or Galβ1,3GalNAc acceptor sugars while the recombinant ST6Gal I transfers NeuAc residues to C6 terminal hydroxyl of Galβ1,4GlcNAc substrates (Figure 1-8).42

Figure 1-7. Common topology of a type II membrane protein.
Figure 1-8. Reactions catalyzed by $\alpha(2\rightarrow6)$ sialyltransferase and $\alpha(2\rightarrow3)$ sialyltransferase.

Aside from common topological features, sialyltransferases do not share any sequence homology with other enzymes in the glycosyltransferase family. However, sequence homology analysis of the sialyltransferase family revealed the existence of several conserved protein motifs within the catalytic domain referred to as L, S, and VS sialylmotifs. The L and S sialylmotifs are located at the center of the lumenal catalytic domain of sialyltransferases and are composed of approximately 48 and 23 amino acid residues, respectively. The VS sialylmotif is located in the C-terminus of sialyltransferases and consists of two highly conserved glutamate and histidine residues that are separated by four amino acid residues. The Paulson laboratory conducted a series of site-directed mutagenesis studies on recombinant rat liver ST6Gal I to investigate the roles of several conserved amino acids in the L & S sialylmotifs. Kinetic data from analysis of ST6Gal I mutant constructs suggested that the L sialylmotif participated in the binding of CMP-NeuAc, while the S sialylmotif participated in the binding of both donor and acceptor substrates. The Paulson group also found that the two invariant cysteine residues present in each of the L and S sialylmotifs for an
intrachain disulfide bond that is essential for retention of catalytic activity and proper conformation of ST6Gal I.\textsuperscript{45}

A closer examination of all known eukaryotic sialyltransferase sequences revealed the presence of another highly conserved motif located between the S and VS sialylmotifs referred to as the aromatic motif.\textsuperscript{46} This motif is comprised of a stretch of four highly conserved mostly aromatic residues. The functional role of these amino acid residues was investigated using site-directed mutagenesis experiments on recombinant human hST3Gal I. The results suggested that the highly conserved histidine (His299) and tyrosine (Tyr300) residues of the aromatic motif are necessary for enzyme activity since their mutation to alanine generated inactive enzymes.

Apart from research involving conserved residues in the sialylmotifs, there is still limited information available concerning the catalytic mechanism and structure of the sialyltransferase family. Previous work in the Horenstein laboratory used radiolabeled CMP-NeuAc and UMP-NeuAc, a weak binding substrate analog, to conduct a series of kinetic isotope effect and pH vs. rate experiments on recombinant rat liver ST6Gal I to elucidate the mechanism of glycosyl transfer. A dissociative mechanism involving a late oxocarbenium ion-like transition state was proposed in the model for sialyltransferase catalysis based on KIE results (Figure 1-9).\textsuperscript{1,47} The pH-rate profile from experiments using UMP-NeuAc and LacNAc as the donor-acceptor substrate pair fits a bell-shaped curve for two ionizable groups with pKa values of 6.2 and 8.9. Further pH-rate experiments and theoretical calculations suggested that glycosyl transfer proceeded via a general acid catalyzed mechanism in which a non-bridging phosphate oxygen on CMP-NeuAc may be protonated to facilitate the loss of CMP.\textsuperscript{1} The kinetic mechanism was
proposed to be steady-state random based on initial velocity, KIE, and isotope trapping experiments.\textsuperscript{1,2}

![Proposed transition-state for sialyltransferase-catalyzed reaction from Horenstein et al.\textsuperscript{47}]

A three dimensional crystal structure of sialyltransferase CstII from \textit{Campylobacter jejuni} was recently reported by Chiu et al.\textsuperscript{48} From the structure, sialyltransferase CstII\textsubscript{Δ32} was categorized under the GT-A fold group because it contained a single Rossmann domain. Aside from this feature, sialyltransferase CstII lacked the conserved ‘DXD’ motif and a bound metal in the active site which are common characteristics among other glycosyltransferases in the GT-A group. Since sialyltransferases do not require a metal cofactor for catalysis,\textsuperscript{49-51} the lack of the conserved ‘DXD’ motif responsible for the binding of a divalent metal cation was not surprising.

In order to observe enzyme-substrate binding interactions in the active site, Chiu et al. crystallized sialyltransferase CstII\textsubscript{Δ32} in the presence of CMP-3FNeuAc, an unreactive substrate analog of CMP-NeuAc (Figure 1-10). The structure of sialyltransferase CstII\textsubscript{Δ32} complexed with CMP-3FNeuAc offers some explanations regarding the nature of sialyl transfer. In the crystal structure, the sialyl moiety of CMP-
3FNeuAc adopted a distorted skew boat conformation which favors formation of the oxocarbenium ion. The leaving-group phosphate was oriented in a pseudo axial position twisted above the plane of the sugar ring allowing the pro-R oxygen on the phosphate to interact with the ring oxygen on NeuAc (Figure 1-10). Cleavage of the glycosidic bond and departure of the CMP moiety was suggested to be facilitated both by the negative-charge buildup on the pro-R phosphate oxygen, and by the hydrogen bonding interactions with the non-bridging phosphate pro-S oxygen and active-site Tyr156 and Tyr162 residues. Mutagenesis experiments using Y156F and Y162F mutants of sialyltransferase CstIIΔ32 resulted in a significant loss in catalytic activity with only one tyrosine residue mutated and a total loss in catalytic activity with both tyrosine residues mutated. Although it is unclear why acid catalysis would be necessary to assist in the departure of a stable monophosphate leaving group, these results indicate that both residues are critical for optimal catalytic efficiency of the CstIIΔ32 transferase mechanism.

![Figure 1-10. Interaction of the ring oxygens of CMP-3FNeuAc with the phosphate oxygens in CstIIΔ32 (left) and interactions of CMP and active site residues (right) from Chiu et al.](image)

Deprotonation of the incoming hydroxyl group of the acceptor sugar was suggested to be catalyzed by His188. The close proximity of His188 at 4.8 Å to the anomeric
carbon in the crystal structure made His188 the only feasible candidate for the role of
general base catalyst; however, this identification is still ambiguous. Based on active-site
comparison studies, His188 is located in a similar position to other catalytic bases
identified in inverting glycosidases. Additionally, the pH optimum of 8.0 for the
CstIIΔ32 catalyzed reaction favors deprotonation of the His188 imidazole, therefore,
allowing it to act as a general base catalyst. A complete loss of transferase activity was
also observed when His188 was mutated to alanine in CstIIΔ32. This same result was
also observed in histidine to lysine/alanine mutagenesis experiments on recombinant
human hST3Gal I and polysialyltransferases, ST8Sia II and IV. Thus, the results
from these experiments reinforce the hypothesis that a histidine residue plays an
important role in the sialyltransferase catalyzed reaction.

Despite the information obtained from this sialyltransferase crystal structure,
bacterial sialyltransferase CstIIΔ32 does not share sequence homology with any
mammalian sialyltransferases, which is where the primary research interest exists. Hence, one can not assume that the mammalian sialyltransferases will adopt the same
structural fold and active-site arrangement as sialyltransferase CstIIΔ32. To date, there
are no three dimensional crystal structures reported for a mammalian sialyltransferase.
This is primarily due to the fact that recombinant mammalian sialyltransferases are more
difficult to overexpress and purify. Sufficient quantities of pure enzyme are arduous to
obtain to conduct crystallization experiments. Furthermore, even if enough pure enzyme
was available to attain a crystal structure, these structures do not provide information
about the reaction’s transition-state structure.
Therefore, other techniques may be used advantageously on mammalian sialyltransferases in order to probe the mechanism of sialyl transfer. Methods such as kinetic isotope effect (KIE) experiments can often provide detailed information on the transition-state structure which will assist in acquiring mechanistic information for the sialyltransferase catalyzed reaction. In this study, several CMP-NeuAc and UMP-NeuAc radioisotopomers were synthesized to investigate the mechanism of sialyl transfer using KIE experiments. The dual-label competitive method was used to measure the KIEs for these radiolabelled substrates with recombinant human placental \( \alpha(2\rightarrow3) \) sialyltransferase and recombinant rat liver \( \alpha(2\rightarrow3) \) sialyltransferase. The data from these experiments will provide an increased understanding of the mechanism of glycosyl transfer with regard to interactions at the phosphate leaving group via \(^{18}\text{O} \) isotopic substitution at the glycosidic O and non-bridging phosphate oxygen atoms.

**Sialyltransferase Inhibitors**

Within the last decade, a burst in the design and synthesis of a variety of sialyltransferase inhibitors occurred due, in part, to their interest as potential therapeutic compounds for the treatment of tumor metastases and immunological diseases. Inhibition studies on purified sialyltransferase also became more feasible in recent years because of the increased commercial availability of recombinant sialyltransferases. Although there have been numerous reported sialyltransferase inhibitors, only the more noteworthy inhibitors will be discussed here.

The most common strategy used toward the design of sialyltransferase inhibitors have been donor substrate based analogs of CMP-NeuAc. The idea behind the development of these inhibitors was to alter functional groups on the sugar or nucleotide portion of the donor substrate, but maintain the basic glycosidic linkage. In 1997,
Schauer and co-workers conducted a series of inhibition studies on recombinant α(2→6) sialyltransferase from rat liver and α(2→3) sialyltransferases from porcine submandibular gland using a variety of different nucleosides, nucleotides, sialic acid and sugar nucleotide analogs as donor substrates. The goal of the study was to identify key structural elements that were essential for inhibition of sialyltransferase. The inhibition studies showed that donor substrate analogs containing a nucleotide monophosphate moiety were the most effective sialyltransferase inhibitors, while the sialic acid analogs displayed little to no inhibition. CMP, CDP, and CTP were natural competitive inhibitors of sialyltransferase with $K_i$ values of 90, 50, and 46 µM, respectively. These inhibition constants are comparable to the $K_m$ value for the natural donor substrate CMP-NeuAc (46 µM). The enhanced inhibitory effect upon addition of one or more phosphate groups was proposed to be caused by their ability to provide a negative charge similar to the carboxylate group of CMP-NeuAc. Moreover, the results from this study suggest that the nucleotide moiety, particularly cytidine monophosphate, is a fundamental structural requirement for high binding affinity of the donor substrate to the enzyme active site.

This information led to the development of more sialyltransferase inhibitors that incorporated the general cytidine or cytidine monophosphate scaffolding in the donor substrate. Schmidt et al. synthesized a series of sialyltransferase inhibitors with cytidine monophosphate linked to quinic acid analogs. These CMP-quinic acid based inhibitors were advantageous to use because they not only included the CMP moiety for high binding affinity, but they also blocked transferase activity by changing the glycosidic bond to a more stable C-glycoside linkage. These compounds were also stable under physiological conditions. Inhibition experiments using CMP-quinic acid as the donor
substrate with ST6Gal I gave a $K_i$ value of 44 µM, which is approximately the same as the $K_m$ value for the natural substrate CMP-NeuAc. With this information in hand, Schmidt and co-workers modified their inhibitor design strategy by synthesizing transition-state analogs that would mimic the oxocarbenium ion-like transition-state proposed for CMP-NeuAc. A few of the transition state analog inhibitors synthesized and tested by Schmidt et al. are shown in Figure 1-11.

These compounds contain a flattened ring with the anomeric carbon trigonal planar to simulate the oxocarbenium ion coplanarity in the transition-state. A methylene group was also added between the anomeric carbon and CMP to model their increased distance in the proposed transition-state structure of CMP-NeuAc. Substitution of the methylene hydrogen with a phosphonate group greatly increased the inhibitory activity of these compounds with $K_i$ values in the nanomolar range. The phosphonate group provided an additional negative charge similar to the carboxylate of CMP-NeuAc. Furthermore, replacing the glycerol side chain on the NeuAc ring with a phenyl group as seen in Figure 1-11, resulted in a 1,000-fold increase in binding affinity to ST6Gal I with a $K_i$ of 29 nM. These results demonstrate the enzyme’s capability to tolerate bulky side chain modifications made to the donor substrate without compromising binding affinity. To date, the phenyl phosphonate compound is the most potent sialyltransferase inhibitor.

Horenstein and co-workers synthesized another unique set of transition state analogs as sialyltransferase inhibitors. This new class of sialyltransferase inhibitor employed an unsaturated bicyclic system with a conjugated carboxylate group to mimic the conformation of the proposed transition-state (Figure 1-12). Additionally, the CMP moiety attached to the bicyclic sytem was kept at an increased distance from the
anomeric carbon to imitate the late transition-state distance proposed for bond cleavage. These compounds were highly efficient inhibitors of sialyltransferase with $K_i$ values in the low micromolar range. Furthermore, substitution of the NeuAc ring with a bicyclic ring illustrates the enzyme’s ability to accept a diverse range of structure changes in the sugar portion of the donor substrate. Recently, Schmidt and co-workers demonstrated that sialyltransferase also exhibit high binding affinities for transition-state analogs with aryl and hetaryl ring systems substituted for the sugar portion. Thus, these studies indicate that the neuraminyl ring only plays a minor role in binding since structure variations to this part of the donor substrate are still tolerated by the enzyme.

Figure 1-11. Structure of CMP-quinic acid and transition-state analogs.
Moreover, these studies have enabled researchers to ascertain that the key components required for sialyltransferase inhibitors are: (i) a planar anomeric carbon; (ii) an increased distance between the anomeric carbon and the leaving group CMP; (iii) at least two negative charges near the cleavage site; and (iv) the cytidine moiety for recognition. Transition-state analogs of CMP-NeuAc have been the most potent of all sialyltransferase inhibitors reported to date. Therefore, information about the transition state acquired from kinetic isotope effect studies may also prove useful toward the development of new sialyltransferase inhibitors.
CHAPTER 2
SYNTHESIS AND CHARACTERIZATION OF SUBSTRATES

Introduction

Cytidine 5’-monophosphate neuraminic acid (CMP-NeuAc) is synthesized by CMP-NeuAc synthetase in many prokaryotic and eukaryotic cells and serves as a key intermediate in the sialyltransferase catalyzed biosynthesis of sialylated oligosaccharides. During catalysis, sialic acid (N-acetylneuraminic acid, NeuAc) is transferred from an activated CMP-NeuAc donor substrate to non-reducing termini of glycoproteins, glycolipids, and oligosaccharide chains. Although sialyltransferases vary in acceptor substrate specificity, all sialyltransferases use CMP-NeuAc as their donor substrate. Thus, information obtained from experiments conducted on recombinant human and rat α(2→3) sialyltransferases, may be applied other members in the sialyltransferase family.

Results and Discussion

Synthesis of CMP-NeuAc isotopomers

In order to probe the mechanism of the sialyltransferase catalyzed reaction, a series of CMP-NeuAc isotopomers were synthesized to perform the desired experiments. These CMP-NeuAc isotopomers either contain one radioactive trace label or a radioactive trace label with several nonradioactive isotopic substitutions. The various sites of isotopic substitution are illustrated in Figure 2-1. The isolated yields are shown in Table 2-1.
Figure 2-1. Structure of labeled CMP-NeuAc. Asterisks denote sites of isotopic substitution.

Table 2-1. CMP-NeuAc isotopomer yields.

<table>
<thead>
<tr>
<th>CMP-NeuAc Isotopomer</th>
<th>Isolated % Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>[9-3H]</td>
<td>74</td>
</tr>
<tr>
<td>[1-3H-N-acetyl]</td>
<td>78</td>
</tr>
<tr>
<td>[1-14C-N-acetyl]</td>
<td>54</td>
</tr>
<tr>
<td>[1-14C-N-acetyl, P^{18}O_{2}]</td>
<td>35</td>
</tr>
<tr>
<td>[1-14C-N-acetyl, 2-^{18}O]</td>
<td>71</td>
</tr>
</tbody>
</table>

Chemical and enzymatic methods were employed to synthesize the various CMP-NeuAc isotopomers (Figure 2-2). The first step of the synthesis required the use of N-acetyl neuraminic acid (NANA) aldolase which was cloned, overexpressed in *E. coli* and purified according to literature procedures.\(^{61-64}\) NANA adolase catalyzes the aldol condensation reaction between pyruvate and N-acetyl mannosamine (ManNAc) to yield NeuAc.\(^{65}\) Isotopomers of NeuAc were obtained by substituting nonradiolabeled ManNAc with the appropriate radiolabeled ManNAc substrate. Several of these radiolabeled ManNAc compounds were purchased commercially, such as, [6-^{3}H] and [1-\(^{14}\text{C-N-acetyl}\)] ManNAc which give [9-^{3}H] and [1-\(^{14}\text{C-N-acetyl}\)] NeuAc, respectively. The [1-\(^{3}\text{H-N-acetyl}\)] NeuAc isotopomer was obtained by synthesizing the [\(^{3}\text{H-N-acetyl}\)] ManNAc starting material via acetylation of D-mannosamine with \(^{3}\text{H}\) acetic anhydride as
per the literature method\textsuperscript{66}. The progress of the radiolabeled NeuAc reactions was monitored by making analytical injections on anion-exchange HPLC and collecting fractions for LSC counting (Figure 2-3). Radiolabeled ManNAc and NeuAc eluted with retention times of 3 min. and 6 min., respectively, on HPLC MonoQ (50-100 mM NH\textsubscript{4}HCO\textsubscript{3} gradient, 15\% methanol, 2 mL/min, A\textsubscript{271}, 2 mL fractions collected).

Figure 2-2. Enzymatic synthesis of N-acetyl neuraminic acid and CMP-NeuAc. Asterisks indicate sites of possible isotopic substitution.

Further isotopic modifications to the radiolabeled NeuAc were made once it was isolated. Deuterium labels were incorporated into the NeuAc at the C3 position by
exchanging the protons with deuterium under alkaline conditions with D$_2$O. This synthesis was performed by Mike Bruner of the Horenstein laboratory as previously described in the literature.$^2$ [3,3'-$^2$H$_2$] CMP-NeuAc was then synthesized with CMP-NeuAc synthetase with the addition of CTP.

![NeuAc Reaction Radioactive Profile](image)

Figure 2-3. Radioactive profile of HPLC fractions from a typical NeuAc reaction. The composition of radioactive ManNAc and NeuAc in the reaction mixture was ~20 % and 80 %, respectively after four days.

**Synthesis of [1-$^{14}$C-$N$-acetyl, 2-$^{18}$O] CMP-NeuAc**

Synthesis of the [1-$^{14}$C-$N$-acetyl, 2-$^{18}$O] CMP-NeuAc isotopomer was achieved by first exchanging the C-2' hydroxyl oxygen on NeuAc via a ring opening mechanism with H$_2$$^{18}$O (95% enrichment) under basic conditions (pH > 9.5) (Figure 2-4).$^{67}$ The enzymatic synthesis with [1-$^{14}$C-$N$-acetyl, 2-$^{18}$O] NeuAc, CTP, and CMP-NeuAc synthetase gave [1-$^{14}$C-$N$-acetyl, 2-$^{18}$O] CMP-NeuAc in 71 % isolated yield after purification by anion-exchange HPLC. The $^{31}$P-NMR spectra showed two peaks at -
4.243 ppm and -4.257 ppm representing the [1-\(^{14}\)C-\(N\)-acetyl, 2-\(^{16}\)O] and [1-\(^{14}\)C-\(N\)-acetyl, 2-\(^{18}\)O] CMP-NeuAc compounds, respectively (Figure 2-5). Integration of the peaks indicated the relative abundance of the [1-\(^{14}\)C-\(N\)-acetyl, 2-\(^{16}\)O] CMP-NeuAc compound to be 25 % and the [1-\(^{14}\)C-\(N\)-acetyl, 2-\(^{18}\)O] CMP-NeuAc compound was 75 %. ESI-MS spectral data was obtained from a parallel nonradiolabeled synthesis of [2-\(^{18}\)O] CMP-NeuAc.

Figure 2-4. Ring opening mechanism for NeuAc.
Figure 2-5. $^{31}$P-NMR of $[1^{-14}C-N\text{-acetyl}, \ 2^{-18}O]$ CMP-NeuAc.

The (-)ESI-MS from the $[2^{-18}O]$ CMP-NeuAc synthesis shows the most abundant ions at $m/z$ 615 [M-H]$^-$ for $[2^{-18}O]$ CMP-NeuAc and $m/z$ 613 [M-H]$^-$ for the $[2^{-16}O]$ CMP-NeuAc compound (Figure 2-6). The [(M-H+Na)-H]$^-$ adduct of $m/z$ 615 was also present at $m/z$ 637. The $m/z$ 615 ion underwent MS/MS to yield the labeled $m/z$ 324 [CMP-H]$^-\text{ion}$. Selected ion monitoring of the ions at $m/z$ 615 ($[2^{-18}O]$ CMP-NeuAc) and $m/z$ 613 ($[2^{-16}O]$ CMP-NeuAc) indicated a distribution of 75.6 % and 24.4 %, respectively.
Figure 2-6. (-) ESI-MS of [2-18O] CMP-NeuAc m/z 615 [M-H]⁻ (top panel), zoom MS/MS of [2-18O] CMP-NeuAc [M-H]⁻ (center panel), and MS/MS dissociation of m/z 615 [M-H]⁻ ion (bottom panel).

**Synthesis of [1-14C-N-acetyl, P18O2] CMP-NeuAc**

Synthesis of the [1-14C-N-acetyl, P18O2] CMP-NeuAc isotopomer was achieved using a multi enzymatic synthesis route to selectively incorporate 18O labels into the non-bridging phosphate oxygens of CMP-NeuAc (Figures 2-6 and 2-7).68
Figure 2-6. Enzymatic synthesis of $[^{18}O_3] \text{CMP}$ from KH$_2[^{18}O_4$. The enzymes used in this synthesis were glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate phosphokinasase (3-PGK) and uridine kinase (UDK).

Figure 2-7. Enzymatic synthesis of $[1-{^{14}C-N-acetyl}, P^{18}O_2] \text{CMP-NeuAc}$ from $[^{18}O_3] \text{CMP}$. 
The first step of the synthesis involved the preparation of KH$_2$P$_{18}$O$_4$ which was synthesized in 74% yield via hydration of PCl$_5$ with H$_2$${}_18$O (95% atom enrichment) followed by the addition of 2 M KOH. This synthesis produced five different phosphate species (P$_{18}$O$_4$, P$_{16}$O$_{18}$O$_3$, P$_{16}$O$_2$$_{18}$O$_2$, P$_{16}$O$_3$$_{18}$O, and P$_{16}$O$_4$) due to the isotopic distribution of the $^{18}$O label. HPLC/(-) ESI-MS and $^{31}$P NMR analysis of KH$_2$P$_{18}$O$_4$ measured the relative isotopic abundance of the five phosphate species to be the following: 80.4% $^{18}$O$_4$, 16.2% $^{16}$O$^{18}$O$_3$, 1.6% $^{16}$O$_2$$^{18}$O$_2$, 0.1% $^{16}$O$_3$$^{18}$O, and 1.7% $^{16}$O$_4$ (Figures 2-8 & 2-9). These results are consistent with the statistical distribution of $^{18}$O for the synthesis of P$_{18}$O$_4$ using 95% atom enriched H$_2$$^{18}$O. The KH$_2$P$_{18}$O$_4$ (80% $^{18}$O$_4$) compound was then used in an enzymatic synthesis with glyceraldehyde-3-phosphate, (G3P), NAD$^+$, ADP, and cytidine to give P$_{18}$O$_3$ CMP in 64% isolated yield after purification on anion-exchange HPLC. The last step in the synthesis of P$_{18}$O$_3$ CMP required the use of uridine kinase (UDK) which was cloned, overexpressed in *E. coli*, and purified by dye affinity chromatography. Enzyme purification yielded 150 units and uridine kinase (23 kDa) was 90-95% pure base on SDS-PAGE analysis (Figure 2-10).
Figure 2-8. (-) ESI-MS of KH$_2^{18}$O$_4$. 
Figure 2-9. $^{31}$P-NMR spectrum of KH$_2$P$^{18}$O$_4$ (1M) in D$_2$O with 4 mM EDTA.
Figure 2-10. 10% SDS-PAGE of purified UDK fractions from Red-A dye affinity column. The sample load for each fraction was 20 µL and the gel was stained with coomassie blue.

The HPLC/MS analysis of P\(^{18}\)O\(_3\) CMP showed four peaks at m/z 324, 326, 328 and 330 corresponding to the P\(^{16}\)O\(_4\) CMP, P\(^{16}\)O\(_3\)^{18}O CMP, P\(^{16}\)O\(_2\)^{18}O\(_2\) CMP, P\(^{16}\)O\(^{18}\)O\(_3\) CMP compounds, respectively (Figure 2-11). An (+) ESI-MS scan measured the relative abundances of the various CMP isotopomers to be: 60% \(^{16}\)O\(^{18}\)O\(_3\), 6.4% \(^{16}\)O\(_2\)^{18}O\(_2\), 1.1% \(^{16}\)O\(_3\)^{18}O, 32.5% \(^{16}\)O\(_4\). The results show an approximate 20% dilution of the \(^{18}\)O label from the initial enrichment of P\(^{18}\)O\(_4\) used in the synthesis. This may be explained by the fact that glyceraldehyde-3-phosphate decomposes at neutral pH to release its phosphate, thus resulting in a dilution of the \([P^{18}O_4^{2-}]\). In response to this result, a shorter incubation time and higher enzyme concentrations were used to help minimize the decomposition of G3P in the reaction mixture. A higher \([P^{18}O_4]\) was also used to reduce unlabeled phosphate incorporation.
Figure 2-11. (+) ESI-MS of P$^{18}$O$_3$ CMP 2 (upper panel) and zoom-MS of the [M+H]$^+$ ions (lower panel).

The [1-14C-N-acetyl, P$^{18}$O$_2$] CMP-NeuAc isotopomer was enzymatically synthesized using P$^{18}$O$_3$ CMP (60 % $^{18}$O$_3$) and [1-14C-N-acetyl] NeuAc. The isolated yield was 35 % after purification on anion exchange HPLC. Since ESI-MS spectral data of a radiolabeled compound could not be obtained, a parallel synthesis was conducted to determine approximate isotopic incorporation for the radiolabeled synthesis. The (+) ESI-MS spectra from the nonradiolabeled synthesis of [P$^{18}$O$_2$] CMP-NeuAc shows the most abundant ions at m/z 619 [M+H]$^+$ for [P$^{16}$O$_2$$^{18}$O$_2$] CMP-NeuAc and m/z 615 [M+H]$^+$ for [P$^{16}$O$_4$] CMP-NeuAc (Figure 2-12). The [M+Na]$^+$ adducts of m/z 619 and m/z 615 were also present at m/z 641 and m/z 637, respectively. The m/z 619 ion underwent MS/MS to yield the labeled m/z 328 [CMP+H]$^+$ ion which in a MS/MS/MS
scan produced the m/z 112 [Cytidine+H]+ ion. Other, less intense ions were detected at m/z 310 and m/z 292 which correspond to fragments of the NeuAc moiety. Selected ion monitoring of the ions at m/z 619 ([P^{16}O_2^{18}O_2] CMP-NeuAc), m/z 617 ([P^{16}O_3^{18}O] CMP-NeuAc), and m/z 615 ([P^{16}O_4] CMP-NeuAc) indicated a distribution of 56.6, 9.2 and 34.2 %, respectively.

Figure 2-12. (+) ESI-MS spectrum of [P^{16}O_2^{18}O_2] CMP-NeuAc.

**Synthesis of UMP-NeuAc Isotopomers**

Previous work conducted in the Horenstein laboratory showed that there is a commitment to catalysis for the CMP-NeuAc donor substrate when bound to the enzyme. In other words, the sialyltransferase catalyzed reaction with CMP-NeuAc donor substrate contains more than one kinetic barrier that is partially rate limiting.
Furthermore, non-chemistry rate limiting steps can mask the full expression of the kinetic isotope effects. To circumvent this problem, UMP-NeuAc, an unnatural “slow” donor substrate for sialyltransferase, was also synthesized to aid in the study of the sialyltransferase catalyzed mechanism. UMP-NeuAc binds more weakly to sialyltransferase and the chemistry step is slower than for CMP-NeuAc as indicated by its higher $K_m$ and lower $k_{cat}$ values.\(^1\) Thus, these factors made UMP-NeuAc an ideal donor substrate analog to use for the desired set of kinetic experiments.

UMP-NeuAc was synthesized with a variety of isotopic substitutions. The locations of these isotopic labels were equivalent to those used in the synthesis of the CMP-NeuAc isotopomers (Figure 2-13). The isolated yields of the UMP-NeuAc isotopomers are shown in Table 2-2.

![Structure of labeled UMP-NeuAc. Asterisks denote sites of isotopic substitution.](image)

Table 2-2. UMP-NeuAc isotopomer yields.

<table>
<thead>
<tr>
<th>UMP-NeuAc</th>
<th>% Isolated Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[9-\text{H}]$</td>
<td>47</td>
</tr>
<tr>
<td>$[1-\text{H}-N\text{-acetyl}]$</td>
<td>33</td>
</tr>
<tr>
<td>$[1-^{14}\text{C}\text{-N\text{-acetyl}}]$</td>
<td>42</td>
</tr>
<tr>
<td>$[1-^{13}\text{C}\text{-N\text{-acetyl}}, P^{18}\text{O}_2]$</td>
<td>30</td>
</tr>
<tr>
<td>$[1-^{14}\text{C}\text{-N\text{-acetyl}}, 2^{18}\text{O}]$</td>
<td>44</td>
</tr>
</tbody>
</table>
Synthesis of the UMP-NeuAc isotopomers was achieved through a chemical deamination reaction using 1 M NaNO₂, pH 3.8 and the appropriately labeled CMP-NeuAc compounds Figure (2-15). This reaction was challenging due to the instability of CMP-NeuAc under acidic conditions. Thus, deamination reactions were carried out at 4 °C to help minimize the decomposition of CMP-NeuAc. The conversion of CMP-NeuAc to UMP-NeuAc also varied depending on how the CMP-NeuAc compounds were purified on anion-exchange HPLC. Deamination reactions using CMP-NeuAc isotopomers purified on anion-exchange HPLC with an ammonium bicarbonate buffer system proceeded slowly, and the conversion of CMP-NeuAc to UMP-NeuAc was ~ 38 % with < 20 % decomposition after 48 hrs at 4 °C (Figure 2-16). This result was explained by considering the concentration of free ammonia present in the solution of CMP-NeuAc after desalting the purified compound with Amberlite IR-120 H⁺. Ammonia assays conducted on the purified and desalted CMP-NeuAc isotopomers estimated the [NH₄⁺] in solution to be ~ 50 mM. The excess NH₄⁺ in the reaction solution slows the progress of CMP-NeuAc deamination by reacting with NaNO₂. This was especially the case when the [CMP-NeuAc] in the reaction was in the micromolar range.

Figure 2-15. Chemical deamination of CMP-NeuAc to UMP-NeuAc by sodium nitrite.
Several ideas were tested to remove more of the NH₄⁺ from the CMP-NeuAc solution such as, filtering the solution through a minicolumn of zeolite, applying the sample to a mini gel filtration column, and desalting the sample again with Amberlite IR-120 H⁺. While these approaches were somewhat effective in removing the [NH₄⁺], the additional purification methods led to a further loss and decomposition of the radiolabeled CMP-NeuAc product. Additional NaNO₂ was also added to the reaction mixture to expedite the deamination of CMP-NeuAc, but this made the desired products more difficult to isolate and purify on anion-exchange HPLC due to the increased complexity of the chromatograms.

![HPLC Chromatogram](image)

**Figure 2-16.** HPLC chromatogram of CMP-NeuAc deamination reaction after 48 hr. The vertical lines represent the beginning and end of fraction collection.

To circumvent this problem, CMP-NeuAc isotopomers slated for deamination were purified on anion-exchange HPLC using a sodium bicarbonate buffer system.

Deamination reactions using the sodium form of CMP-NeuAc resulted in a mixture consisting of 11% CMP-NeuAc, 66% UMP-NeuAc, 3% CMP, and 20% UMP after 30
hours at 4 °C (Figure 2-17). These yields were calculated from peak integration values from an HPLC chromatogram. This reaction was advantageous because it allowed the synthesis of UMP-NeuAc isotopomers in higher yield without significant decomposition of the starting material and the final product. This aspect was essential in order to obtain enough UMP-NeuAc isotopomer to carry out the desired set of experiments. Furthermore, unreacted CMP-NeuAc could be recovered during purification of UMP-NeuAc and recycled for use in experiments requiring a CMP-NeuAc isotopomer.

Figure 2-17. HPLC chromatogram of CMP-NeuAc deamination after 30 hrs incubation. The vertical lines represent the beginning and end of fraction collection.

**Experimental**

**Materials**

Reagents and buffers were purchased from Sigma and Fisher and used without further purification. Recombinant rat liver α(2→3) and α(2→6) sialyltransferase was purchased from Calbiochem. The ^18^O water (95% atom enrichment) was purchased from
Isotec and Medical Isotopes, Inc. N-acetyl D-mannosamine isotopomers ([1-14C-N-acetyl] and [6-3H]) were purchased from Moravek and American Radiolabelled Chemicals. Glyceraldehyde-3-phosphate (G3P), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [EC 1.2.1.12] and 3-phosphoglycerate phosphokinase (3-PGK) [EC 2.7.2.3] were purchased from Sigma. Nucleoside monophosphate kinase (NMK) [EC 2.7.4.4] was purchased from Roche Applied Science. Liquid scintillation fluid (ScintiSafe 30 %) was purchased from Fisher. The \textit{E. coli} expression plasmid pWV200B harboring the \textit{E. coli} CMP-NeuAc synthetase gene [EC 2.7.7.43] was a generous gift from Dr. W. F. Vann at the National Institutes of Health.

**Instrumental**

A Rainin HPLC system consisting of a HPXL binary pump and a model UV-1 detector was used. HPLC separations were performed on a Mono Q HR 10/10 anion exchange column (Amersham-Pharmacia) monitored at 271 nm. Data collection was achieved on a personal computer using the Star Workstation Version 6.2 software (Varian Inc.). A Rainin-Dynamax fraction collector (model FC-1) was used to collect eluent samples from the HPLC. A Packard 1600 TR instrument was used for liquid scintillation counting. Mass spectrometry (LC-MS) was performed on a ThermoFinnigan (San Jose, CA) LCQ in electrospray ionization (ESI) mode. The system was interfaced with an Agilent (Palo Alto, CA) 1100 binary pump HPLC system consisting of an Applied Biosystems Model 785A programmable absorbance detector set at 254 nm. HPLC separations for LC-MS were performed on a Phenomenex (Torrace, CA) Synergy 4u Hydro-RP 80A C18 column (mobile phases = 0.5 % HOAc in water/0.5 % HOAc in methanol). \(^{31}\)P-NMR spectra were acquired on a 300 MHz Mercury NMR spectrometer. \textit{E. coli} cells were lysed using a French pressure cell with Carver hydraulic press.
Centrifugation was performed with a Sorvall RC 5B centrifuge. A Labconco CentriVap Concentrator (speedvac) was used to concentrate small volume samples.

**Synthesis of [³H-N-acetyl] ManNAc**

[¹³H-N-acetyl] ManNAc was synthesized using a procedure adapted from that of Roseman *et. al.* Freshly prepared Dowex 1 x 8-200 mesh (CO₃²⁻) (250 mg damp) and D-mannosamine-HCl (12.3 mg, 56 µmol) was suspended in 250 µL of water and carefully added to the ampoule containing [³H] acetic anhydride (5 mCi, s.a.-100 mCi/mmol, 50 µmol). The reaction was stirred in an ice bath for 4 hours. The aqueous solution was removed from the reaction solution and passed over an amberlite IR-120 H⁺ minicolumn. The eluate was collected in a 25 mL round bottomed flask. The reaction ampule was washed three times with 1 mL of water and the solution was passed over the amberlite column after each wash. Water (1 mL) was used to wash the amberlite minicolumn twice more. The 25 mL flask containing the eluate was fitted to a short path distillation apparatus with a 10 mL receiving flask. The solution was refluxed twice and then cooled to room temperature. The mixture was then concentrated to dryness *in vacuo*, keeping the bath temperature below 55 °C. The pot residue was twice resuspended in 2 mL of water and reconcentrated *in vacuo*. The residue was dissolved in 500 µL of water and [¹³H-N-acetyl] ManNAc was purified from the reaction mixture via multiple injections onto a HPLC C18, 1 x 30 cm (5 % methanol (v/v), 95 % water, A₂₂₀ nm, 1 mL/min). The [¹³H-N-acetyl] peak (r.t. 10 min) was collected after each injection. The [¹³H-N-acetyl] ManNAc fractions were combined, the solution was concentrated to dryness in vacuo, and the product was resuspended in 1 mL of water. The product contained 150 µCi for an isolated yield of 6 %.
Synthesis of [1-^3H-N-acetyl] NeuAc and [1-^3H-N-acetyl] CMP-NeuAc

[1-^3H-N-acetyl] D-mannosamine (30 µCi) was concentrated to dryness in vacuo using the speedvac. Phosphate buffer, pH 7 (40 mM, 100 µL) containing 1 mg/mL BSA and 1 mg/mL NaN₃ was added to the 1.5 mL reaction tube. Sodium pyruvate (10 mg, 1 mmol) and 2 units of NANA aldolase were also added to the reaction tube. The reaction mixture was placed at room temperature for four days and judged to be >80% complete by LSC counting of reaction aliquots analyzed by HPLC (2 mL HPLC Mono-Q fractions, 15% methanol (v/v), 500 mM NH₄HCO₃, pH 8.0, 10 – 20% salt gradient, 2 mL/min). The retention times for [1-^3H-N-acetyl] ManNAc and [1-^3H-N-acetyl] NeuAc were 3 min and 6.5 min, respectively. The reaction mixture was concentrated to dryness in vacuo using the speedvac and resuspended in 120 µL of 10 mM HEPES, pH 7.5 buffer containing NeuAc (10 mM, 0.4 mmol) and CTP (15 mM, 1 mmol). CMP-NeuAc synthetase (3 units) and 4 µL of 2.5 M MnCl₂ were also added to the reaction tube. The mixture was incubated at 37 °C for 8 hours and the judged to be >80% complete by LSC counting of reaction aliquots analyzed by HPLC (2 mL HPLC Mono-Q fractions, 15% methanol (v/v), 75 mM NH₄HCO₃, pH 8.0, isocratic, 2 mL/min). The product was purified by HPLC on a Mono-Q column. The [1-^3H-N-acetyl] CMP-NeuAc fraction was collected, desalted with Amberlite IR-120 H⁺, concentrated to dryness in vacuo, and the material was resuspended in 300 µL of diH₂O. The purified product contained 15 µCi for a yield of 78%. This procedure was also used to synthesize other isotopomers of NeuAc and CMP-NeuAc by using the appropriately radiolabeled precursors.
Cloning, Overexpression and Purification of N-acetylneuraminic Acid Aldolase [EC 4.1.3.3]

The N-acetylneuraminic acid aldolase (NANA aldolase) gene was amplified from *E. coli* K12 genomic DNA using PCR (upper primer-5’-ATGGCAACGAATTTACGTGGCGTAA-3’ and lower primer 5’-TCACCCGCCTCTTTGCTCAACTGC-3’). The gel purified PCR product was ligated into the pETBlue-1 vector and transformed into NovaBlue competent cells for plasmid amplification. The plasmid was purified using the QIAprep Spin Miniprep Kit (Qiagen) and subsequently used in a transformation reaction with Tuner™ (DE3)pLacI competent cells. IPTG induction of a 2 L culture of the Tuner™ (DE3)pLacI cells harboring the recombinant plasmid resulted in overexpression of the target enzyme. N-acetylneuraminic acid lyase was purified following the published protocol and was judged 90-95 % pure based on SDS-PAGE analysis.

Overexpression of CMP-NeuAc Synthetase [EC 2.7.2.43]

The original pWV200B plasmid harboring the CMP-NeuAc synthetase gene was given as a generous gift from Dr. W. F. Vann. The plasmid was transformed into *E. coli* JM109 cells via electroporation and the cells were plated onto luria broth agar plates containing 60 µg/mL ampicillin. Overnight incubation of the plates at 37 °C yielded ~200 colonies. Several colonies were selected from the plates with a sterile toothpick and used to inoculate a culture tube containing 5 mL of luria broth supplemented with 100 µg/mL ampicillin. The culture was grown in a 37 °C shaking incubator (200 rpm) until an O.D.₆₀₀nm = 0.8 – 1.0. The 5 mL culture was then used to inoculate a 2 L culture of luria broth containing 100 µg/mL ampicillin. The culture was grown in a 37 °C shaking incubator (200 rpm) until an O.D.₆₀₀nm = 0.6 – 0.8. The culture was induced with IPTG
(1 mM final concentration) for 10 hours in a 37 °C shaking incubator to overexpress CMP-NeuAc synthetase. The cells were harvested via centrifugation at 5000 rpm, 4 °C, for 30 min. The cells were resuspended in 20 mL of purification buffer (10 mM HEPES, pH 7.0 containing 1 mM EDTA, 10 mM MgCl2, and 1 mM PMSF) and lysed using a French pressure cell and Carver hydraulic press (2 runs). The supernatant was separated from cellular debris via centrifugation at 8000 rpm, 4 °C for 30 min. CMP-NeuAc synthetase was purified from the supernatant by Red-A (Millipore) dye affinity column chromatography (2.5 x 7 in) using a linear salt gradient from 0-1 M KCl in purification buffer. Fractions were analyzed for protein using the Bradford assay method and for CMP-NeuAc synthetase activity using the published assay.73,74 CMP-NeuAc synthetase containing fractions were combined and concentrated in an Amicon concentrator to a final protein concentration of >10 mg/mL. The protein solution was then saturated with 80 % (NH4)2SO4 and the precipitate was stored at 4 °C until further use. The yield was ~ 60 units and the CMP-NeuAc synthetase was 90-95 % pure based on SDS-PAGE analysis.

**Cloning, Overexpression and Purification of Uridine Kinase [EC 2.7.1.48]**

The uridine kinase (UDK) gene71 was amplified from *E. coli* K12 genomic DNA using PCR (upper primer-5’-ATGACTGACCAGTCTCACCAGTAGTGCG-3’ and lower primer –5’-AAGCTTATTCAAAGAAGCAGTGGCG-3’). The PCR product was gel purified using the QIAquick Gel Extraction Kit (Qiagen) and ligated into the pETBlue-1 vector (Novagen). The recombinant plasmid was transformed into NovaBlue (Novagen) competent cells and the plasmid was purified using QIAPrep Spin Miniprep Kit (Qiagen). Transformation of Tuner™(DE3)pLacI competent cells with the construct and IPTG-induced overexpression of a 2 L culture yielded the target enzyme. Uridine
kinase was purified by Red-A (Millipore) dye affinity column (2.5 x 7 in) using a linear salt gradient from 0-1 M KCl (0.1 M Tris-HCl, pH 7.8, 4 mM EDTA). Fractions were analyzed for protein using the Bradford assay method and for UDK activity using the published assay. UDK containing fractions were combined and concentrated in an Amicon concentrator to a final volume of 2 mL. The yield was ~ 150 units and the UDK was 90-95 % pure based on SDS-PAGE analysis.

**Synthesis of 75 atom % [1-14C-N-acetyl, 2-18O] CMP-NeuAc**

A mixture of N-acetyl neuraminic acid (2 mg, 6 µmol) and [1-14C-N-acetyl] neuraminic acid (15 µCi) was dissolved in 200 µL of 1.9 mM glycine, 0.5 M MnCl₂, pH 9.5 buffer. The solution was concentrated to dryness in vacuo and the material was resuspended in 300 µL of H₂18O (95 % enrichment). The reaction mixture was placed at 37 ºC for 16 hrs. Cytidine-5-triphosphate (15 mg, 28 µmol) was then added to the reaction tube along with CMP-NeuAc synthetase (2 units). The pH of the reaction mixture was adjusted to pH 7.5 as necessary with 5 N NaOH. The reaction was incubated at 37 ºC for 8 hrs and judged to be >75 % complete by LSC counting of 2 mL HPLC Mono-Q fractions (15 % methanol (v/v), 75 mM NH₄HCO₃, pH 8.0, isocratic, 2 mL/min, A₂71). The product was purified by HPLC on a Mono-Q column. The [1-14C-N-acetyl, 2-18O] CMP-NeuAc fraction was collected, desalted with Amberlite IR-120 H⁺, concentrated to dryness in vacuo, and the material was resuspended in 300 µL of diH₂O. The purified product contained 12 µCi for a yield of 71 %.

**Synthesis of KH₂P¹⁸O₄**

KH₂P¹⁸O₄ was synthesized using a method similar to that of Risley et al. The ¹⁸O water (95 % atom enrichment) (300 µL, 15 µmol) was added drop wise via syringe to a two necked flask containing phosphorus pentachloride (428 mg, 2 mmol). The PCl₅
was weighed in a dry box. Once removed, it was immediately placed on a Schlenk line under constant flow of N\textsubscript{2} (g). This was done to reduce H\textsubscript{2}\textsuperscript{16}O contamination. The reaction was stirred at 0 °C for 1 hr under constant flow of dry N\textsubscript{2} (g). The flask was then warmed to room temperature and heated in a water bath at 100 °C for 30 min. The remaining reaction solution was cooled to room temperature and ~ 2 mL of deionized water was added. The solution was titrated to pH 5 with 2 M KOH and KH\textsubscript{2}P\textsuperscript{18}O\textsubscript{4} was precipitated from solution by addition of 95 % ethanol. The precipitate was collected by concentration under reduced pressure.

**Synthesis of P\textsuperscript{18}O\textsubscript{3} CMP**

For the synthesis of P\textsuperscript{18}O\textsubscript{3} CMP a solution of KH\textsubscript{2}P\textsuperscript{18}O\textsubscript{4} (1M, pH 7.0) was made from which 250-350 µL was mixed with glyceraldehyde-3-phosphate (60 µL, 2 mM), β-NAD (60 µL, 3 mM), ADP (40 µL, 1 mM), MgSO\textsubscript{4} (50 µL, 2.8 mM), glycine (50 mM), cytidine (40 µL, 2.5 mM), GAPDH (2 units), 3-PGK (1 unit), and uridine kinase (2 units) in a 1.5 mL microfuge tube and incubated at room temperature for 10 hrs. Special care was taken to minimize contamination by unlabeled phosphate initially present in some of the reagents used in the reaction. Thus, ADP was freshly prepared and 3-PGK was dialyzed against 0.5 M Tris-HCl, pH 7.5 buffer to remove the orthophosphate storage buffer provided by the manufacturer. The reaction solution was filtered through a microcon filtration unit (Millipore, MWCO 10 kDa) to remove enzymes and P\textsuperscript{18}O\textsubscript{3} CMP was purified from the filtrate using isocratic, anion exchange HPLC (75 mM NH\textsubscript{4}HCO\textsubscript{3}, pH 8.5, 15% methanol, 2 mL/min.). The P\textsuperscript{18}O\textsubscript{3} CMP containing fractions were pooled and desalted with Amberlite IR 120-H\textsuperscript{+} cation-exchange resin. The solution was concentrated to dryness in vacuo and resuspended in 300 µL of deionized water.
Synthesis of [1-\textsuperscript{14}C-N-acetyl, P\textsuperscript{18}O\textsubscript{2}] CMP-NeuAc

The [1-\textsuperscript{14}C-N-acetyl, P\textsuperscript{18}O\textsubscript{2}] CMP-NeuAc was synthesized using a method adapted from that of Ichikawa et al.\textsuperscript{77} The P\textsuperscript{18}O\textsubscript{3} CMP purified from above was concentrated to dryness in \textit{vacuo} and used in an enzymatic reaction with ATP (5 µmol), PEP monosodium salt (10 µmol), MnCl\textsubscript{2} (10 µmol), MgCl\textsubscript{2} (10 µmol), NMK (2 units), PK (500 units) and 700 µL of HEPES buffer (0.2 M, pH 7.5). The reaction mixture was incubated for 24 h at 25 °C and then filtered through a microcon filtration device (Millipore, MWCO 10 kDa) to remove enzymes. This step is necessary to prevent the in-situ recycling of P\textsuperscript{18}O\textsubscript{3} CMP and subsequent dilution of \textsuperscript{18}O labels resulting from the decomposition of CMP-NeuAc in the following synthetic step. [1-\textsuperscript{14}C-N-acetyl] NeuAc (10-25 µCi) and CMP-NeuAc synthetase (3 units) were added to the filtrate and the reaction was incubated at 37 °C for 6 h. CMP-NeuAc isotopomers were purified using isocratic, anion exchange HPLC (75 mM NH\textsubscript{4}HCO\textsubscript{3}, 15 % methanol, pH 8.0, 2 mL/min). CMP-NeuAc fractions were pooled, desalted with Amberlite IR 120-H\textsuperscript{+} cation-exchange resin and concentrated \textit{in vacuo} as previously described.

Synthesis of UMP-NeuAc

CMP-NeuAc was purified on HPLC Mono Q (15 % methanol (v/v), 75 mM NaHCO\textsubscript{3}, pH 8.0, isocratic, 2 mL/min). The CMP-NeuAc fraction was collected, desalted with Amberlite IR-120 H\textsuperscript{+}, concentrated to dryness \textit{in vacuo}, and the material was resuspended in 300 µL of diH\textsubscript{2}O. The solution of CMP-NeuAc (2 mM, 370 µmols) was made 1 N with NaNO\textsubscript{2} and adjusted to pH 3.5 – 4.0 with 1 N HCl while on ice. The reaction mixture was placed at 4 °C for 30 hours. The reaction proceeds to 65 – 70 % completion with less than 20 % hydrolysis of starting material in this time. This method
was also used to synthesize the various UMP-NeuAc isotopomers by using the appropriate CMP-NeuAc isotopomers.
CHAPTER 3
PURIFICATION AND KINETIC CHARACTERIZATION OF RECOMBINANT
HUMAN ALPHA (2→3) SIALYLTRANSFERASE IV

Introduction

Complex carbohydrates and polysaccharides are biosynthesized in living systems via an intricate pathway of membrane-bound and secreted proteins. Many of these carbohydrate moieties are added by glycosyltransferases to specific biomolecules as the “finishing touches” during post-translational events in the Golgi apparatus of cells. The biosynthesis of sialylated glycans are governed by a unique group of enzymes in the glycosyltransferase family known as sialyltransferases. Sialyltransferase are membrane-bound proteins that transfer sialic residues (NeuAc) from activated CMP-NeuAc to specific acceptor oligosaccharides, glycolipids, and glycoproteins during post-translational modification. The addition of these sialic acid residues assists in the regulation of a myriad of cellular processes that are of significant biological importance. This knowledge has prompted the need to study the sialyltransferase family of enzymes in order to probe the functional roles of sialic acid ‘capped’ biomolecules required for many key biological processes.

According to the most recent reviews on the sialyltransferase family, approximately 20 distinct cDNA sequences for sialyltransferases have been identified and cloned from various mammalian and bacterial sources. The most widely studied sialyltransferases are the α(2→6) and α(2→3) sialyltransferases from rat liver which are commercially available in recombinant forms. In this study, three truncated forms of
recombinant human α(2→3) sialyltransferase (h23STGal IV, EC 2.4.99.4), which lacked the first 61 amino acids coding for the NH2-signal anchor of the open reading frame, were overexpressed in Spodoptera frugiperda (Sf-9) insect cells using a baculovirus expression vector. The NH2-terminal signal anchor sequence was replaced in all three enzymes with a cleavable canine insulin signal peptide to produce a soluble, catalytically active secreted protein.\textsuperscript{78,79} The other two recombinant forms of h23STGal IV contained a His\textsubscript{6x}-tag sequence at the N- and C- termini of the sialyltransferase sequence. The three enzymes were overexpressed, purified via affinity chromatography, and used to conduct the desired sets of kinetic experiments.

### Results and Discussion

**Overexpression and Purification of Recombinant Human α(2→3) Sialyltransferase Isoforms**

The cDNA sequence of human α(2→3) sialyltransferase from placenta has been reported\textsuperscript{79}, which greatly facilitated the primer design for PCR amplification of the recombinant sialyltransferase isoforms. The cDNA clones encoding the h23STGal IV gene which lacked the transmembrane domain and the creation of the pFastBacHTa vector harboring the recombinant h23STGal IV gene were prepared by Dr. Nicole Horenstein. To produce the enzyme in a more readily purified form, the N-terminal His\textsubscript{6x} tag sequence located downstream from the strong polyhedron (pPolh) promoter in the pFastBacHTa vector was removed via a restriction digest with \textit{RsrII} and \textit{BamHI} endonucleases and replaced with a 114 bp insert containing the sequence coding for the cleavable canine pancreas insulin signal peptide.\textsuperscript{78} This allowed the h23STGal IV enzyme to become a secreted protein. Two other recombinant h23STGal IV constructs containing N- and C-terminal His\textsubscript{6x} tags, additional to the insulin signal peptide, were
prepared in a similar manner by Bronson Anatao.\textsuperscript{80} These recombinant His\textsubscript{6x} tag h23STGal IV constructs were created to simplify the standard purification procedure. One construct contained an N-terminal His\textsubscript{6x} tag located between the insulin signal peptide and the catalytic domain of recombinant h23STGal IV, while the other construct contained a C-terminal His\textsubscript{6x} tag located at the end of the recombinant h23STGal IV catalytic domain sequence. Figure 3-1 illustrates the order of the insulin peptide sequence, recombinant h23STGal IV gene sequence, and the His\textsubscript{6x} tag peptide sequence for each of the three constructs described above. The recombinant pFastBacHTa vectors harboring the three enzyme constructs were subsequently used to create the recombinant baculoviruses following the protocols outlined in the BEVS manual supplied by the manufacturer.\textsuperscript{81}

Figure 3-1. Diagram of the recombinant h23STGal IV constructs. The insulin signal peptide sequence is represented in yellow, the truncated h23STGal IV sequence is represented in blue and the His\textsubscript{6x}-tag sequence is represented in purple.
A baculovirus expression system using insect host cells was chosen to overexpress recombinant h23STGal IV because of its reported success in overexpressing other catalytically active enzymes in the glycosyltransferase family. Baculoviruses are one of the most prominent viral pathogens affecting the insect species. This system is widely used because of its capability for expressing high levels of recombinant protein and because of its ability to provide the eukaryotic post-translational modifications required to produce active enzymes of this type. Several glycosyltransferases have also been successfully overexpressed in active form with other eukaryotic host cells such as *Saccharomyces cerevisiae* and methylotrophic yeast *Pichia pastoris*. Attempts to overexpress recombinant mammalian sialyltransferases in *E. coli* resulted in the production of an insoluble, inactive form of the enzyme. This outcome is presumably because *E. coli* lacks the capability to sufficiently glycosylate mammalian sialyltransferases during post translational modification in the Golgi apparatus to allow for proper folding of the enzymes into an active form. In our hands, attempts to express the sialyltransferase gene in *P. pastoris* were also unsuccessful.

Recombinant baculovirus was generated by first cloning the truncated h23STGal IV sialyltransferase constructs into the pFastBacHTa donor plasmids and transforming the recombinant vectors into competent DH10Bac *E. coli* (Figure 3-2). Site-specific transposition was used to insert the recombinant sialyltransferase constructs into bacmid DNA provided in the *E. coli* host cells. Recombinant bacmid DNA was purified from *E. coli* and used to transfect *Sf-9* insect cells. Recombinant baculovirus particles were generated after incubating the tranfection mixture with the insect cells for several days. Baculovirus stocks were amplified several times by infecting fresh *Sf-9* cultures grown to
a density of $2 \times 10^6$ cells/mL until a viral titer of $1 \times 10^7$ to $1 \times 10^8$ pfu/mL was achieved. Large scale expression of the recombinant h23STGal IV was achieved by infecting liter cultures of $Sf-9$ insect cells with the amplified recombinant baculovirus stocks. Recombinant h23STGal IV was expressed and secreted into the culture medium during the late phase of the viral life cycle. The average h23STGal IV activity accumulated in the cell supernatant of a 1 L $Sf-9$ cell culture was (2-3 U/L) after 72 hours post infection with recombinant baculovirus. Purification of recombinant Ins-h23STGal IV was achieved using sepharose CDP-hexanolamine affinity column chromatography. Since sepharose CDP-hexanolamine resin is not commercially available, the CDP-hexanolamine ligand was synthesized and attached to a CNBr activated sepharose 4B resin as previously described in the literature (Figure 3-3). Table 3-1 summarizes the data obtained from the purification of a 370 mL scale $Sf-9$ expression of Ins-h23STGal IV on sepharose CDP-hexanolamine. The results shown in Table 3-1, Figures 3-4 and 3-5 were obtained in collaboration with Jeremiah D. Tipton of the Horenstein laboratory.
Figure 3-2. General scheme for the generation of recombinant baculoviruses and protein expression with the BAC-TO-BAC expression system.

Figure 3-3. Structure of CDP-Hexanolamine affinity ligand synthesized for the purification of recombinant Ins-h23STGal IV enzyme.
Table 3-1. Recombinant Ins-h23STGal IV purification table.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Activity (mU)</th>
<th>Yield (%U)</th>
<th>Concentration (mg/mL)</th>
<th>Total (mg)</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Supernatant</td>
<td>370</td>
<td>823</td>
<td>100</td>
<td>0.080</td>
<td>29.6</td>
<td>0.029</td>
</tr>
<tr>
<td>Amicon Ultrafiltration</td>
<td>80</td>
<td>767</td>
<td>94</td>
<td>0.440</td>
<td>35.2</td>
<td>0.022</td>
</tr>
<tr>
<td>CDP-hexanolamine pooled fractions</td>
<td>28</td>
<td>227</td>
<td>28</td>
<td>0.050</td>
<td>1.40</td>
<td>0.162</td>
</tr>
<tr>
<td>Concentration</td>
<td>5</td>
<td>159</td>
<td>20</td>
<td>0.241</td>
<td>1.20</td>
<td>0.132</td>
</tr>
</tbody>
</table>

The crude supernatant was concentrated to a volume of 80 mL with an Amicon Ultrafiltration unit fitted with a polyethersulfone membrane (MWCO 10 kDa) prior to purification on the sepharose CDP-hexanolamine column. This step was necessary to reduce the volume of supernatant applied to the affinity column in order to expedite the purification process. Glycerol (20 % v/v) and Triton CF-54 (0.01 % v/v) were added to the crude supernatant to help stabilize recombinant h23STGal IV during concentration and purification. The crude supernatant was loaded onto the sepharose CDP-hexanolamine affinity column and the column was washed with three column volumes of purification buffer containing 300 mM β-lactose prior to eluting the column with a KCl step gradient. This step was necessary to elute gp64, a predominant baculovirus membrane glycoprotein responsible for virus-cell fusion, that was found to co-elute with recombinant h23STGal IV in earlier pilot purification trials. Gp64 is believed to act as an acceptor substrate for recombinant h23STGal IV. Since β-lactose serves as an acceptor substrate for recombinant h23STGal IV, enzyme-gp64 binding interactions can be disrupted by washing the column with high concentrations of β-lactose.
Purified recombinant h23STGal IV was obtained in 28 % yield upon elution of the affinity column with a KCl step gradient. An activity assay and a protein assay were performed on select fractions to determine the elution profile of the enzyme from the affinity column (Figure 3-4). The final yield of purified recombinant h23STGal IV was 20 % with a specific activity of 0.132 U/mg after concentrating the pooled fractions with an Amicon Ultrafiltration unit. Purified recombinant h23STGal IV gave three bands in the SDS-PAGE gel with sizes of ~40 kDa, 37 kDa, and 35 kDa, which represent three different glycoforms of the enzyme (Figure 3-5). These results are consistent with the results from a protein sequence analysis of recombinant h23STGal IV of MS experiments where four potential asparagine N-linked glycosylation sites were identified following a general protein sequence motif of N/X/S (X represents any amino acid). Digestion experiments performed by Jeremiah D. Tipton of the Horenstein laboratory on the purified recombinant Ins-h23STGal IV with PNGase, gave one protein band on an SDS-PAGE gel at 33 kDa, which is the expect size for the deglycosylated protein.

After several purification trials, it was found that recombinant Ins-h23STGal IV appeared to be sensitive to the concentration step following purification as shown in Table 3-1 by the decrease in specific activity from 0.162 to 0.132 U/mg. The reason for this result is unclear, but one explanation may be that the enzyme denatures during the concentration step. Surfactants such as Triton CF-54 and Tween-80 have been shown to help stabilize enzyme activity during the purification of other recombinant sialyltransferases. Therefore, Triton CF-54 (0.01 % v/v) was added to the enzyme buffer to stabilize the enzyme and help minimize the loss of activity during this step. Additionally, a significant loss in enzyme activity was observed when a
concentrated sample of recombinant Ins-h23STGal IV was diluted and reconcentrated. In this case, the enzyme may denature upon dilution and appears to be incapable of renaturing into active form when concentrated again. As a result, this step was avoided during the purification of recombinant Ins-h23STGal IV.

Figure 3-4. Typical elution chromatogram of recombinant Ins-h23STGal IV from a sepharose CDP-hexanolamine affinity column. Solid squares represent protein concentration and open diamonds represent activity.
Figure 3-5. 10% SDS-PAGE of purified recombinant Ins-h23STGal IV. Lane 1, MW standard; Lane 2, Lane 3, and Lane 4 are 10 µL, 20 µL, and 30 µL loads of a TCA precipitation of purified Ins-h23STGal IV, respectively (left gel). SDS-PAGE of purified recombinant Ins-h23STGal IV digested with PNGase. Lane 1, MW standard; Lane 2, 30 µL load of a TCA precipitation of purified Ins-h23STGal IV and Lane 3 is 30 µL load of PNGase digested Ins-h23STGal IV. The gels were stained with coomassie blue.

Ni²⁺-NTA affinity chromatography was employed for the purification of NtermHis- and CtermHis-h23STGal IV enzymes. Glycerol (20% v/v) and Triton CF-54 (0.01% v/v) were added to the crude supernatant to stabilize the enzymes during the purification. A 300 mM β-lactose wash was also performed as described previously to remove gp64 prior to eluting the column with an imidazole step gradient. Purified recombinant NtermHis-h23STGal IV and CtermHis-h23STGal IV were obtained in 47% and 32% yield, respectively, after eluting Ni²⁺ the affinity column with an imidazole step gradient. An activity assay and a protein assay were performed on select fractions to determine the elution profile of the enzymes from the affinity column (Figure 3-6). The final yield of purified recombinant NtermHis-h23STGal IV and CtermHis-h23STGal IV
was 12 % and 43 % with specific activities of 0.022 U/mg and 0.161 U/mg, respectively after concentration and dialysis (Tables 3-2 & 3-3). The results shown in Tables 3-2 and 3-3 and in Figure 3-6 were obtained in collaboration with Jeremiah D. Tipton of the Horenstein laboratory. Purified recombinant NtermHis-h23STGal IV and purified recombinant CtermHis-h23STGal IV gave three bands in the SDS-PAGE gel with sizes of ~ 42 kDa, 41 kDa, and 40 kDa, which represent three different glycoforms of the enzyme (Figure 3-7). The size of the enzyme glycoforms for the recombinant NtermHis- and CtermHis-h23STGal IV are larger than for the recombinant Ins-h23STGal IV enzyme glycoforms. Since the addition of the His$_{6x}$ tag peptide sequence in these enzymes would only add an additional 1000 Da to the size of the recombinant Ins-h23STGal IV glycoforms, the reason for the larger sizes is unclear. One explanation for the size discrepancy may be that the presence of the His$_{6x}$ tag alters the addition of glycan chains during post translational modification to produce recombinant sialyltransferase with a higher degree of glycosylation.

Table 3-2. Recombinant NtermHis-h23STGal IV purification table.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Activity (mU)</th>
<th>Yield (%U)</th>
<th>Concentration (mg/mL)</th>
<th>Total (mg)</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Supernatant</td>
<td>460</td>
<td>419</td>
<td>100</td>
<td>0.240</td>
<td>70.5</td>
<td>0.004</td>
</tr>
<tr>
<td>Ni$^{2+}$-NTA Pooled Fractions</td>
<td>30</td>
<td>190</td>
<td>47</td>
<td>0.050</td>
<td>1.50</td>
<td>0.126</td>
</tr>
<tr>
<td>Concentration &amp; Dialysis</td>
<td>4.5</td>
<td>50</td>
<td>12</td>
<td>0.500</td>
<td>2.25</td>
<td>0.022</td>
</tr>
</tbody>
</table>
Table 3-3. Recombinant CtermHis-h23STGal IV purification table.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Activity (mU)</th>
<th>Yield (%)</th>
<th>Concentration (mg/mL)</th>
<th>Total (mg)</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Supernatant</td>
<td>470</td>
<td>560</td>
<td>100</td>
<td>0.150</td>
<td>71</td>
<td>0.008</td>
</tr>
<tr>
<td>Ni$^{2+}$-NTA Pooled Fractions</td>
<td>50</td>
<td>181</td>
<td>32</td>
<td>0.03</td>
<td>1.5</td>
<td>0.121</td>
</tr>
<tr>
<td>Concentration &amp; Dialysis</td>
<td>5.5</td>
<td>243</td>
<td>43</td>
<td>0.273</td>
<td>1.5</td>
<td>0.161</td>
</tr>
</tbody>
</table>

The recombinant NtermHis-h23STGal IV enzyme also appeared to be sensitive to the concentration step after purification as shown in Table 3-2 by the decrease in specific activity from 0.126 to 0.022 U/mg. Concentration and dialysis of this enzyme without the presence of a surfactant such as Triton CF-54, resulted in a total loss of enzyme activity. Therefore, as for the recombinant Ins-h23STGal IV, Triton CF-54 (0.01 % v/v) was added to the enzyme buffer prior to concentration and dialysis. The recombinant CtermHis-h23STGal IV enzyme, however, did not lose activity during the concentration and dialysis step as seen by the increase in specific activity from 0.121 to 0.161 U/mg. This may be due to the overall stability of the recombinant CtermHis-h23STGal IV enzyme in comparison to the recombinant NtermHis-h23STGal IV. It is reasonable to consider that the position of the His$_{6x}$ tag on the N- or C-terminus may change the protein folding structure in a manner that would make recombinant NtermHis-h23STGal less stable than CtermHis-h23STGal IV during concentration. Additionally, the position of the N-term His$_{6x}$ tag could also interfere with the addition of glycan chains during post translational that are essential for enzyme stability and activity. This effect has been observed for other Hi$_{6x}$-tagged glycosyltransferases. The specific activity of the
recombinant CtermHis-h23STGal IV enzyme was similar to that of recombinant Ins-h23STGal IV, but the yield was higher than for the recombinant Ins-h23STGal IV.

Figure 3-6. Typical elution chromatogram of recombinant NtermHis-h23STGal IV and CtermHis-h23STGal IV from a Ni$^{2+}$-NTA affinity column. Solid squares represent protein concentration and open diamonds represent activity.

Figure 3-7. 10% SDS-PAGE gel of purified recombinant CtermHis-h23STGal IV NtermHis-h23STGal IV. Lane 1, TCA precipitation of purified CtermHis-h23STGal IV; Lane 2, MW Standard; Lane 3, TCA precipitation of purified NtermHis-h23STGal IV. The gel was stained with coomassie blue.
Kinetic Characterization of Recombinant h23STGal IV Isoforms

The kinetic parameters obtained for the three recombinant isoforms of human α(2→3) sialyltransferase using CMP-NeuAc and α-lactose as the donor-acceptor substrate pair were estimated by fitting the initial velocity kinetic data to the Michaelis-Menten equation using a least squares analysis in Sigma Plot ver. 9.0 (Figures 3-8 – 3-10). Recombinant Ins-h23STGal IV, NtermHis-h23STGal IV, and CtermHis-h23STGal IV enzymes were concentrated a second time to specific activities of 0.05, 0.012, and 0.042 U/mg, respectively as determined by activity assays prior to their use in steady state kinetic experiments in order to provide concentrated enough enzyme samples. The measured kinetic parameters were similar to those obtained for a wild type α(2→3) sialyltransferase purified from human placenta (Table 3-4).97 The CMP-NeuAc K_m values for NtermHis-h23STGal IV and Ins-h23STGal IV were 82 ± 5 µM and 74 ± 8 µM, respectively. These K_m values were on the same order of magnitude as the wild type α(2→3) sialyltransferase from human placenta and were comparable to the literature K_m value of 74 µM obtained for recombinant rat liver α(2→3) sialyltransferases expressed from insect cells.56 However, the CMP-NeuAc K_m value obtained for CtermHis-h23STGal IV was ~3.5 fold higher than for the NtermHis-h23STGal IV and Ins-h23STGal IV enzymes. This reason for this is unclear and literature kinetic data on other C-terminal His_{6x} tagged sialyltransferases have not been reported. However, a few hypotheses for this result are that the addition of the CtermHis_{6x} tag on recombinant h23STGal IV either interferes with the binding site for CMP-NeuAc or that it changes the structural fold of the enzyme in a manner that alters CMP-NeuAc binding. The α-lactose K_m values for all three recombinant isoforms of human α(2→3) sialyltransferase were similar to each other and for the wild type enzyme within experimental error.97
Table 3-4. Kinetic parameters for sialyltransferase isoforms.

<table>
<thead>
<tr>
<th>Recombinant Enzyme</th>
<th>CMP-NeuAc</th>
<th>α-Lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (µmol·min$^{-1}$·mg$^{-1}$)</td>
</tr>
<tr>
<td>WT h23STGal IV$^{97}$</td>
<td>63</td>
<td>-</td>
</tr>
<tr>
<td>Ins-hST3Gal IV</td>
<td>82 ± 5</td>
<td>0.072 ± 0.002</td>
</tr>
<tr>
<td>NtermHis-hST3Gal IV</td>
<td>74 ± 8</td>
<td>0.008 ± 0.0003</td>
</tr>
<tr>
<td>CtermHis-hST3Gal IV</td>
<td>267 ± 20</td>
<td>0.041 ± 0.002</td>
</tr>
</tbody>
</table>

The $V_{max}$ value obtained for Ins-ST was 0.072 ± 0.004 µmol/(min·mg) which is slightly lower than the 0.12 ± 0.01 µmol/(min·mg) $V_{max}$ reported for the wild type human placenta α(2→3) sialyltransferase when CMP-NeuAc and α-lactose were used as the donor-acceptor substrate pair.$^{97}$ This difference in $V_{max}$ values was not large and reflects the observed loss in enzyme specific activity after concentrating the enzyme. The $V_{max}$ values obtained for CtermHis-h23STGal IV and NtermHis-h23STGal IV were 3-6 fold lower than that of the wild type sialyltransferase. However, these values were similar to the specific activities obtained for these enzymes after the second concentration step described above. If the recombinant enzymes were not concentrated after purification, than their $V_{max}$ values may have been more comparable to that reported for wild type h23STGal IV enzyme.
Figure 3-8. Michaelis-Menten plots for recombinant Ins-h23STGal IV with varied [CMP-NeuAc] and constant [α-lactose] (top panel) and with varied [α-lactose] and constant [CMP-NeuAc] (bottom panel).
Figure 3-9. Michaelis-Menten plots for recombinant NtermHis-h23STGal with varied [CMP-NeuAc] and constant [α-lactose] (top panel) and with varied [α-lactose] and constant [CMP-NeuAc] (bottom panel).
Figure 3-10. Michealis-Menten plots for recombinant CtermHis-h23STGal IV with varied [CMP-NeuAc] and constant [α-lactose] (top panel) and with varied [α-lactose] and constant [CMP-NeuAc] (top panel) and for with (lower panel).
Conclusions

Overall, the recombinant NtermHis-h23STGal IV and CtermHis-h23STGal IV enzymes were significantly easier to purify than the recombinant Ins-h23STGal IV enzyme. Furthermore, the Ni$^{2+}$-NTA resin can readily obtained from commercial sources thus allowing for larger scale expressions of recombinant enzyme to be purified more rapidly. This is not the case for the recombinant Ins-h23STGal IV enzyme which requires a tedious synthesis of a sepharose CDP-Hexanolamine affinity resin prior to purification. The protein expression scale is also limited by the amount of affinity resin synthesized. Of the three enzymes, recombinant CtermHis-h23STGal IV enzyme gave the best results with the highest purification yield and specific activity. This enzyme also had comparable kinetic parameters to the recombinant Ins-h23STGal IV enzyme except for the slightly larger $K_m$ value obtained for CMP-NeuAc.

Experimental

Materials and Methods

Reagents and buffers were purchased from Sigma and Fisher and used without further purification. The restriction enzymes, *E. coli* strains JM109 & ER2925, Klenow, Large Fragment (DNA Polymerase I), and T4 DNA ligase were purchased from New England Biolabs. Shrimp Alkaline Phosphatase (SAP) was purchased from Roche Molecular Biology. The Wizard® Plus Minipreps Kit and dNTPs were purchased from Promega. The QIAquick® Nucleotide Removal Kit and QIAquick® Gel Extraction Kit were purchased from Qiagen. The BCA Protein Assay Kit was purchased from Pierce. The BAC-TO-BAC Baculovirus Expression System (BEVS), *Spodoptera frugiperda* (Sf 9) insect cells, BACPACK™ Baculovirus Rapid Titer Kit, and DH10 BAC *E. coli* competent cells were purchased from Invitrogen. Human placental cDNA was obtained
from Clontech. Primers for cloning and PCR analysis were obtained from Integrated DNA Technologies. The protocol for recombinant virus preparation is found in Invitrogen’s instruction manual for BEVS version D April 6, 2004 (www.invitrogen.com). The sepharose CDP-hexanolamine affinity column was prepared as per the literature. N-acetyl neuraminic acid (NANA) aldolase [EC 4.1.3.3] used in the synthesis of [9-3H] neuraminic acid (NeuAc) was cloned, overexpressed in E. coli and purified according to literature procedures. The E. coli expression plasmid pWV200B haboring the E. coli CMP-NeuAc synthetase gene [EC 2.7.7.43] used for the synthesis of all CMP-NeuAc substrates was a generous gift from Dr. W. F. Vann at the National Institutes of Health. Radioactive samples for sialyltransferase activity determination and kinetic experiments were analyzed with a Packard 1600 TR liquid scintillation analyzer. DNA sequencing was performed at the University of Florida ICBR DNA sequencing core.

**Preparation of pFastBacHTaInsulin/h23STGal IV (Ins-h23STGal IV)**

MAX EFFICIENCY DH10BAC E. coli cells transformed with the pFASTBAC plasmid containing the recombinant h23STGal IV gene were previously prepared in our lab by Dr. Nicole Horenstein. A canine insulin construct located upstream from the h23STGal IV was also cloned into the recombinant plasmid to allow for secretion of the enzyme into Sf-9 insect cell media. The construct, pFastBacHTaInsulin/h23STGal IV, was submitted for DNA sequencing to confirm the presence of the canine insulin secretion peptide insert. The protocol for baculovirus preparation is found in the manual BAC-To-BAC® Baculovirus Expression Systems provided by Invitrogen. The recombinant bacmid DNA was purified from E. coli using mini-prep procedures and
subsequently used in the transfection of Sf-9 insect cells to produce recombinant baculovirus particles.

**Preparation of pFastBacHTaInsulin/NtermHis$\text{_{6x}}$-tag-h23STGal IV Plasmid (NtermHis-h23STGal IV)**

A fresh glycerol stock of *E. coli* JM109 cells harboring the pFastBacHTaInsulin/h23STGal IV plasmid was used to inoculate a 5 mL luria broth culture supplemented with 100 µg/mL ampicillin. The culture was grown overnight at 37 ºC (200 rpm) and the plasmid was subsequently isolated using the Wizard® Plus Minipreps kit. The isolated plasmid was digested for 12 hours at 37 ºC with BamHI. Clean-up of the enzymatic digestion was performed using the QIAquick® Nucleotide Removal Kit. The BamHI digested plasmid was digested for 6 hours at 37 ºC with StuI. After thermally inactivating StuI at 65 ºC for 20 min., the doubly digested plasmid was agarose gel purified using the QIAquick® Gel Extraction Kit. The 5’ ends generated by digestion were dephosphorylated using SAP.

The entire NtermHis$_{6x}$ tag insert was created by allowing two complimentary primers to anneal and then be extended by Klenow, Large Fragment. Primers were mixed in equal volumes (2.5 µL) of NtermHISFor_Upper (5’- GGTAGGCCCTGCGCATCAAAGCGGATGCTGGAGATGGGAGCTATCAAGAACCTCACGTCC-3’), (50 µM, 0.80 µg/µL) and NtermHisRev_Lower (5’- AGCAGGGCTGCTCCTGCCACCTGGAGGAGCGGCACGGCTTCTTTTCTCGCC-3’), (50 µM, 0.81 µg/µL) and heated at 90 ºC for 10 minutes. After allowing the mixture to equilibrate to room temperature, it was brought up to a total volume of 20 µL containing 11.16 µL deionized water, 420 µM of a dNTP mixture, 10 mM Tris-HCl pH 7.5, 5 mM MgCl$_2$, 7.5 mM dithiothreitol, and 5 U of Klenow, Large Fragment. This
reaction mixture was then allowed to incubate in a 25 °C water bath for 80 minutes. After inactivating the polymerase at 75 °C for 20 minutes, the mixture was cooled to room temperature and then doubly digested with *BamHI* and *Stul* at 37 °C for 9 hours.

Following heat inactivation of *Stul*, a clean-up of the enzymatic reaction was performed using the QIAquick® Nucleotide Removal Kit. The NtermHis<sub>6x</sub> tag insert was then ligated into the *BamHI/Stul* sites of the pFastBacHTaInsulin/h23STGal IV vector. The new construct, pFastBacHTaInsulin/NtermHis<sub>6x</sub>-tag-h23STGal IV, was submitted for DNA sequencing to confirm the presence of the NtermHis<sub>6x</sub> tag insert. Isolation of bacmids and generation of baculovirus stocks followed the BEVS protocol.<sup>81</sup>

**Preparation of pFastBacHTaInsulin/CtermHis<sub>6x</sub>-tag-h23STGal IV Plasmid (CtermHis-h23STGal IV)**

The pFastBacHTaInsulin/h23STGal IV plasmid was purified from *E. coli* strain ER2925 (dcm<sup>−</sup>) using the Wizard® Plus Minipreps kit. This step was necessary to produce plasmid that could be restricted with Dam or Dcm<sup>−</sup> sensitive restriction enzymes such as *Eco0109I*. The isolated plasmid was digested with *Eco0109I* for 10 hours at 37 °C. Clean-up of the enzymatic digestion was performed using the QIAquick® Nucleotide Removal Kit. The *Eco0109I* digested plasmid was digested for 8 hours at 37 °C with *XhoI*. After thermally inactivating *Eco0109I* and *XhoI* at 65 °C for 20 min., the doubly digested plasmid was agarose gel purified using the QIAquick® Gel Extraction Kit. The 5’ ends generated by digestion were dephosphorylated using SAP.

The CtermHis<sub>6x</sub> tag insert was created following a similar procedure to that of the NtermHis<sub>6x</sub> tag insert. Primers were mixed in equal volumes (2.3 µL) of CtermHISFor_Upper (5’-)

GGTAGGCCCCTGGCCATTAAGCGGATGCTGGAGATGGGAGCTATCAAGAACCT
CACGTCC-3′), (50 µM, 0.882 µg/µL) and CtermHisRev_Lower (5′-
AGCCTCGAGTTAGTGATGGTGATGGTGATGACCGCCGAAGGACGTGAGGTTC
TTGATAGC-3′), (50 µM, 0.917 µg/µL) and heated at 90 °C for 10 minutes. After
allowing the mixture to equilibrate to room temperature, it was brought up to a total
volume of 20 µL containing 11.5 µL deionized water, 463 µM of a dNTP mixture, 10
mM Tris-HCl pH 7.5, 5 mM MgCl2, 7.5 mM dithiothreitol, and 5 U of Klenow, Large
Fragment. This reaction mixture was then allowed to incubate in a 25 °C water bath for 2
hrs. After inactivating the polymerase, the mixture was cooled to room temperature and
then digested with Eco0109I for 16 hours at 37 °C. The enzymatic reaction was cleaned
up using the QIAquick® Nucleotide Removal Kit. The clean mixture was digested with
XhoI at 37 °C for 8 hours. XhoI was then thermally inactivated. The CtermHis6x tag
insert was then ligated into the Eco0109I/XhoI sites of the
pFastBacHTaInsulin/h23STGal IV vector. The new construct,
pFastBacHTaInsulin/CtermHis6x-tag-h23STGal IV, was submitted for DNA sequencing
to confirm the presence of the CtermHis6x tag insert. Isolation of bacmids and generation
of baculovirus stocks followed BEVS protocol.81

**Amplification of Recombinant Baculovirus Plasmids**

Recombinant baculovirus stocks, as prepared following the BEVS protocol, were
amplified four times until a titer of $1 \times 10^7 - 3 \times 10^8$ pfu/mL was obtained.81 For
amplification and expression, cell cultures (50 mL) containing $2 \times 10^6$ cells/mL in 250
mL borosilicate shaker flasks were infected with 3 mL of $1.7 \times 10^8$ pfu / mL Ins-
h23STGal IV, 3 mL of $2.1 \times 10^8$ pfu / mL NtermHis-h23STGal IV, or 3 mL of $2.7 \times 10^8$
pfu / mL CtermHis-h23STGal IV.
Expression and Purification of Ins-h23STGal IV

A 1 L culture of Sf-9 insect cells (20, 250 mL shaker flasks containing 50 mL of culture each) at concentrations of 2 x 10^6 cells/mL were infected with 3 mL of amplified baculovirus stocks (~2 x 10^8 pfu/mL) harboring the recombinant sialyltransferase expression constructs. The cultures were incubated for 70 hrs at 27 ºC after which the cultures were combined and centrifuged at 14,000 rpm, 4 ºC, for 30 min to pellet the cells. The supernatant was harvested and concentrated to 50 -100 mL using a 200 mL Amicon Ultrafiltration unit equipped with a polyethersulfone membrane (MWCO 10 kDa). This concentration step was only done for the Ins-h23STGal IV expressions in order to expedite the purification process on the CDP-hexanolamine affinity column.

The concentrated supernatant was brought to 20 % (v/v) glycerol and 0.01 % (v/v) Triton CF-54 and applied to the sepharose CDP-hexanolamine affinity column (1.7 x 12 cm) previously equilibrated with purification buffer (50 mM MES, pH 6.8 buffer containing 300 mM β-lactose, 20 % (v/v) glycerol, and 0.01 % (v/v) Triton CF-54) at 4 ºC. The column was then washed with at least three column volumes of purification buffer to remove gp64 glycoprotein from recombinant Ins-h23STGal IV. Ins-h23STGal IV was purified from the supernatant by eluting the column with a KCl step gradient of 50 mM, 250 mM, and 400 mM KCl in 50 mM MES, pH 6.8 buffer with 20 % (v/v) glycerol and 0.01 % (v/v) Triton CF-54. Fractions were analyzed for protein and activity using the Bradford assay method the published activity assay.56,73 Fractions containing activity were pooled, dialyzed, and concentrated. Bradford and bicinchoninic acid (BCA) assays were used to estimate protein concentration.73,101
Expression and Purification of NtermHis-h23STGal IV and CtermHis-h23STGal IV Isoforms

A 1 L culture of Sf-9 insect cells (20, 250 mL shaker flasks containing 50 mL of culture each) at concentrations of 2 x 10⁶ cells/mL were infected with 3 mL of amplified baculovirus stocks (~2 x 10⁸ pfu/mL) harboring the recombinant sialyltransferase expression constructs. The cultures were incubated for 70 hrs at 27 °C after which the cultures were combined and centrifuged at 14,000 rpm, 4 °C, for 30 min to pellet the cells. The clarified supernatant was brought to 20 % (v/v) glycerol and 0.01 % (v/v) Triton CF-54 and loaded onto a Ni²⁺-NTA column (2 x 10 cm) previously equilibrated with 50 mM MES, pH 6.8 buffer containing 5 mM imidazole, 100 mM KCl, 20 % (v/v) glycerol, and 0.01 % (v/v) Triton CF-54 at 4 °C. After loading the supernatant onto the column, the column was washed with at least three column equivalents of 50 mM MES, pH 6.8 buffer containing 300 mM β-lactose, 5 mM imidazole, 100 mM KCl, 20 % (v/v) glycerol, and 0.01 % (v/v) Triton CF-54, followed by a 5 column equivalent wash with 50 mM MES, pH 6.8 buffer containing 5 mM imidazole, 100 mM KCl, 20 % (v/v) glycerol, and 0.01 % (v/v) Triton CF-54. NtermHis-h23STGal IV and CtermHis-h23STGal IV enzymes were eluted from the column using imidazole step gradient of 50 mM, 75 mM, and 120 mM imidazole in 50 mM MES, pH 6.8 buffer with 100 mM KCl, 20 % (v/v) glycerol, and 0.01 % (v/v) Triton CF-54. Fractions were analyzed for protein using the Bradford assay method and for sialyltransferase activity using the published assay. Fractions containing activity were pooled, dialyzed, and concentrated. Bradford and BCA assays were used to estimate protein concentration.
Sialyltransferase Enzyme Activity Assays

All activity assays reported for the recombinant h23STGal IV isoforms were performed using methods described by Paulson et al.\textsuperscript{56,90} The assay contained a mixture of \([9-^3\text{H}]\) CMP-NeuAc (100-170 µM, 20,000 cpm, s.a. = 4-7 µCi/µmol) and 235 mM α-lactose in 50 mM MES, pH 6.8 buffer containing 0.01 % (v/v) Triton CF-54, and 1 mg/mL BSA. A 10 µL aliquot from a selected sialyltransferase containing sample was incubated with 10 µL of the \([9-^3\text{H}]\) CMP-NeuAc/α-lactose mixture for the appropriate amount of time to limit the consumption of CMP-NeuAc to < 10 %. The reaction mixture was quenched with 500 µL of 5 mM inorganic phosphate buffer, pH 6.8 and then applied to 2.5 cm Dowex 1 x 8, 200 mesh (PO\textsubscript{4}\textsuperscript{2-}) mini-columns equilibrated with 5 mM Pi, pH 6.8.\textsuperscript{56,102} Reactions were eluted with 3.5 mL of 5 mM Pi buffer, pH 6.8 into liquid scintillation vials. Liquid scintillation vials were counted for 5 min., and all tubes were cycled through the counter 4-5 times to obtain an accurate measurement of the amount of radioactive product. The definition of a unit of activity is the amount CMP-NeuAc converted to sialyl-lactose per minute. The activity reported was obtained by correcting the observed velocities with the obtained kinetic parameters and the concentrations of substrate employed, as fit to the following bi-substrate equation:

\[
\nu = \left(\frac{v_{\text{max}} \times [A] \times [B]}{K_{mA} x K_{mB} + K_{mA} \times [B] + K_{mB}[A] + [A] x [B]}\right)
\]

\textbf{Steady State Kinetics for Recombinant h23STGal IV Isoforms}

The kinetic parameters for the recombinant human α(2→3) sialyltransferase isoforms with CMP-NeuAc and α-lactose as the donor-acceptor pair were estimated by varying the CMP-NeuAc concentration while holding α-lactose at a near-saturating concentration, and by vary the α-lactose concentration and holding CMP-NeuAc at a
near-saturating concentration. Reactions were conducted at 37 °C for 12 min in 50 mM MES, 0.2 mg/mL BSA, 0.05 % (v/v) Triton CF-54, pH 7.5 buffer with a final volume of 100 µL. Each reaction contained 100,000 cpm of [9-3H] CMP-NeuAc diluted to the required specific activity. The apparent $K_m$ value for CMP-NeuAc was obtained by using 30-300 µM of CMP-NeuAc with 0.5 mM of $\alpha$-lactose, and for $\alpha$-lactose, using 50-500 mM of $\alpha$-lactose with 300 µM CMP-NeuAc. The reactions were initiated by the addition of 8-12 µg of recombinant enzyme. Aliquots of 20 µL were removed at 3, 6, 9, and 12 min and quenched in 500 µL of 5 mM phosphate, pH 6.8 buffer. The product was quantified using the Dowex column methodology as described above. Samples were counted in a liquid scintillation counter for 10 min., and all tubes were cycled through the counter 6-10 times to obtain an accurate measurement of the amount of radioactive product. The kinetic parameters obtained for the three recombinant isoforms of human $\alpha(2\to3)$ sialyltransferase using CMP-NeuAc and $\alpha$-lactose as the donor-acceptor substrate pair were estimated by fitting the kinetic data to the Michaelis-Menten equation using a least squares analysis in Sigma Plot ver. 9.0.

Michaelis-Menten Equation:

$$v = \frac{V_{\text{max}} [S]}{K_m + [S]}$$  \hspace{1cm} \text{eq. 3-2}
CHAPTER 4
KINETIC ISOTOPE EFFECT STUDIES ON RECOMBINANT SIALYLTRANSFERASES

Introduction

Kinetic isotope effects (KIEs) serve as a valuable technique to elucidate the transition-state structure of organic and enzymatic reactions. Knowledge about the transition-state structure of an enzyme catalyzed reaction is significant in that it offers detailed information about the reaction mechanism in a way that enzyme crystal structures are unable to provide. In this study, a series CMP-NeuAc and UMP-NeuAc donor substrate radioisotopomers were synthesized to probe the mechanism of sialyl transfer using KIE experiments. By measuring a variety of KIEs at different positions on the donor substrate, one can gain valuable information about various aspects of the transition-state structure which will assist in acquiring mechanistic information for the sialyltransferase catalyzed reaction. The dual-label competitive method was used to measure the KIEs for the various donor substrate radioisotopomers with recombinant human placental $\alpha(2\rightarrow3)$ sialyltransferase, recombinant rat liver $\alpha(2\rightarrow3)$ sialyltransferase, and recombinant rat liver $\alpha(2\rightarrow6)$ sialyltransferase. The data from these experiments will provide an increased understanding of the mechanism of glycosyl transfer with regard to interactions at the phosphate leaving group via $^{18}$O isotopic substitution at the glycosidic O and non-bridging phosphate oxygen atoms for a family of enzymes. Additionally, this data may prove useful toward the development of new
sialyltransferase inhibitors that are based on the transition-state structure of the donor substrate.

**Kinetic Isotope Effect (KIE) Background**

**Isotope Effect Theory**

Isotope effects are simply explained as the perturbation of the reaction rate (kinetic isotope effect, KIE) or of the reaction equilibrium constant (equilibrium isotope effect, EIE) resulting from an isotopic substitution at one position in a reaction molecule. While the description of isotope effects seems straightforward, the interpretation of isotope effects can be quite complicated. In general, isotope effects are expressed as a ratio of rate constants where the rate constant for the light molecule (k_L) is divided by the rate constant for of the heavy (k_H).

The initial theoretical calculations for isotope effects and their use to investigate chemical reaction mechanisms was published by Bigeleisen and Mayer in 1947.\(^1\) The work presented by Bigeleisen and Mayer on the calculation of equilibrium isotope effects established the foundation for the field of isotope effects. The Bigeleisen equation for equilibrium isotope effects is shown in equation 4-1.

\[
K_1/K_2 = \text{MMI} \cdot \text{EXC} \cdot \text{ZPE} \quad \text{eq. 4-1}
\]

For this equation, K_1 and K_2 represent the equilibrium constants for the two isotopic species being measured. The MMI term includes moments of inertia and the combined molecular mass. The EXC term accounts for the isotope effect on the molecules if they exist in excited vibrational states. Lastly, the ZPE term denotes the isotope effect resulting from differences in vibrational zero-point energy.\(^2\)
The Bigeleisen equation for equilibrium isotope effects was later extended to include isotope effects on reaction rates (KIEs). Kinetic isotope effects are related to equilibrium isotope effect theory via the transition-state theory. The basis of the theory is centered on the supposition that the reactant and the transition-state are in equilibrium. The rate of the reaction can then be derived from the transition-state theory as the difference in free energy when going from the ground state of the reaction to the transition-state as expressed in equation 4-2.

\[ k = \frac{(kT/h) \exp(-\Delta G^\ddagger/R T)}{1} \]  

In this equation \( k \) represents Boltzmann’s constant, \( h \) denotes Planck’s constant, \( T \) is the temperature in Kelvin, \(-\Delta G^\ddagger\) corresponds to the activation free energy, and \( R \) is the ideal gas constant.

Under the assumptions provided in the transition-state theory, the Bigeleisen equation can be applied to kinetic isotope effects with a slight modification to the normal 3N-6 vibrational modes for the ground-state EXC and ZPE terms. In the transition state, one normal mode turns into a reaction coordinate mode with an imaginary frequency, \( v_L \). The reaction coordinate mode accounts for the motion along the reaction coordinate since the transition-state can convert either back to reactants or forward to products. Thus, transition states have 3N-7 frequencies with one imaginary frequency. The equation for KIE is expressed in eq. 4-3. The mathematical expansions of the individual terms in the
Bigeleisen equation for KIE are shown in Figure 4-1. In this equation \( u \) is equal to \( h\nu/KT \).

\[
KIE = v_L^i/v_H^i \cdot MMI \cdot EXC \cdot ZPE
\]

\[\text{eq. 4-3}\]

\[
\text{MMI} = \left( \frac{M_1^+}{M_2^-} \times \frac{M_2^+}{M_1^-} \right)^{3/2} \left( \frac{A_1^+B_1^+C_1^+}{A_2^+B_2^+C_2^+} \times \frac{A_2B_2C_2}{A_1B_1C_1} \right)^{1/2}
\]

\[
\text{EXC} = \prod_{i}^{3N^i-7} \frac{1}{1-e^{-u_{i(1)}}} \prod_{i}^{3N-6} \frac{1-e^{-u_{i(1)}}}{1-e^{-u_{i(2)}}}
\]

\[
\text{ZPE} = \prod_{i}^{3N^i-7} \frac{\left(\frac{1}{2}\right)^{u_{i(2)}}}{e^{(1/2)u_{i(1)}}} \prod_{i}^{3N-6} \frac{\left(\frac{1}{2}\right)u_{i(1)}}{e^{(1/2)u_{i(2)}}}
\]

Figure 4-1. Expanded terms of the Bigeleisen equation.

Isotope effects, whether they are EIE or KIE, generally originate from the ZPE term. Since molecules of biological interest are normally large, the contribution to the isotope effect from the translational, rotational, and excited vibrational energies is usually small and therefore insignificant. Consequently, the zero-point energy typically becomes the dominating factor of the isotope effect. Most of the isotope effect stems from the differences in zero-point energy between isotopomers of the initial and final states when either going from reactant to product or in going from the ground-state to the transition-state. When considering kinetic isotope effects, the contribution to the isotope effect from the zero-point energy factor is determined by the change in force constants to the
isotopically substituted atom upon moving from the ground-state to the transition-state. For example, if the bond to the isotopically substituted atom becomes looser in the transition-state, then a normal (>1) KIE will be observed. In this case, the zero-point energy term decreases because the force constant of this particular bond diminishes in the transition-state. This change to the isotope effect is illustrated in Figure 4-2 where the potential energy well becomes wider in going from the ground-state to the transition-state on the reaction coordinate. Conversely, if the bond to the isotopically substituted atom becomes tighter in the transition-state, then an inverse (<1) KIE will be observed. The zero-point energy term in this situation increases because the force constant of this bond becomes larger in the transition-state. This is depicted in Figure 4-3 where the potential energy well becomes narrower in going from the ground-state to the transition-state on the reaction coordinate. Lastly, if the bond to the isotopically substituted atom does not change upon going from the ground-state to the transition-state, than a KIE of unity will be observed. These general changes to the isotope effect may be summarized by the first rule in isotope chemistry which states that the light isotopic molecule prefers a looser bonding state where the restrictions to vibration are lower.³

Since isotope effects arise from changes in force constants and zero-point energies on the bond attached to the isotopically substituted atom, they are therefore local effects and can only extend a couple of bond distances. As a result, isotope effects are categorized into different types depending on the location of the isotopic substitution to the reaction center. The two major types of isotope effects are primary and secondary effects which will be discussed in greater detail below.
Figure 4-2. Free energy diagram depicting the looser potential energy wells in the transition-state resulting in a normal (>1) isotope effect from Lowry et al.4

Figure 4-3. Free energy diagram depicting the looser potential energy wells in the transition-state resulting in an inverse (<1) isotope effect from Lowry et al.4
Primary Isotope Effects

Primary kinetic isotope effects occur when the isotopically substituted atom experiences either bond formation or bond cleavage in the transition-state. In general, primary isotope effects are larger than secondary isotope effects because the bond changes taking place at the site of isotopic substitution translate into higher changes in ZPE. The measurement of carbon heavy atom primary isotope effects for molecules of biological interest is commonly achieved by isotopically substituting a carbon reaction center with a $^{14}$C or $^{13}$C atom. The size of the primary isotope effect is contingent upon the type of symmetry that occurs around the reaction center in the transition-state. If the reaction center is symmetrical at the transition-state, than the symmetric stretching vibration in the transition-state will be the major contributor to the primary isotope effect. This is explained when one considers the different vibrational modes between the ground-state and the transition-state. The vibrational modes that exist in the reactant are the bending and stretching vibrations. In the transition-state, the vibrational modes include the bending vibration, the symmetric stretching vibration, and the vibrational mode that becomes the reaction coordinate. If the bending vibrations are similar between the ground-state to the transition-state, these vibrations cancel to leave only the symmetric stretching vibration. As a result, the symmetric stretching vibration becomes the main contributor to the primary isotope effect. The symmetric stretching vibration is depicted in Figure 4-4 where the isotopically substituted atom “C” is being moved from “A” to “B”. In a symmetrical transition-state, atom C is motionless and the symmetric stretching vibration involves only A and B. The isotopically substituted atom, therefore, does not significantly contribute to the vibrational frequency in the transition-state because atom C will not have a zero-point energy difference. Symmetrical transition-
states occur in associative $S_N2$ reactions. Associative $S_N2$ reactions normally have larger primary $^{14}C$ isotope effects with values in the range of 1.08-1.15.

On the other hand, in an asymmetrical transition-state, the isotopically substituted atom contributes more to the primary isotope effect because it keeps some of the symmetrical stretching vibrational frequency. In this case, the symmetrical stretching vibrational frequency attributed to atom C will partially cancel the zero-point energy difference in the ground-state to give a decreased kinetic isotope effect. Asymmetrical transition-states are characteristic for dissociative $S_N1$ reactions. Dissociate $S_N1$-like reactions typically have smaller primary $^{14}C$ kinetic isotope effects with values in the range of 1.02-1.05.

Secondary Isotope Effects

Secondary isotope effects occur when a bond to the isotopically substituted atom is neither broken or formed in the transition-state. Secondary isotope effects are typically smaller than primary isotope effects and they do not directly report on bond breaking and

![Diagram of symmetric and asymmetric transition-states](image)
bond formation in going from the ground-state to the transition-state. The two common
types of secondary isotope effects are $\alpha$- and $\beta$-secondary isotope effects.

In $\alpha$-secondary isotope effects, the site of isotopic substitution is at the reaction
center. These effects generally occur when the reaction center undergoes a change in
hybridization or a change in nonbonding interactions. A normal isotope effect is
observed if the hybridization state changes from sp$^3$ to sp$^2$ in the transition-state as
shown in Figure 4-2. Conversely, if the hybridization state changes from sp$^2$ to sp$^3$ in
the transition-state, an inverse isotope effect will be observed as depicted in Figure 4-3.
Unlike primary carbon isotope effects, $\alpha$-secondary isotope effects are unable to
differentiate $S_N1$ and $S_N2$ type reaction mechanisms.

In $\beta$-secondary isotope effects, the site of isotopic substitution is adjacent to the
reaction center. $\beta$-secondary isotope effects result when hyperconjugation occurs
between the isotopically substituted atom, such as $\beta$-deuterium (C-H/D), and the electron
deficient p-orbital at the adjacent carbon in a dissociative transition-state. A normal
isotope effect is generally observed for $\beta$-secondary isotope effects because the C-H/D
bond loosens in the transition-state as shown in Figure 4-2. The magnitude of the $\beta$-
secondary isotope effect relies on the degree of positive charge build-up in the transition-
state and the dihedral angle ($\theta$) between the empty p-orbital on the reaction center and the
bond to the isotopically substituted atom. Maximal effects are seen for dihedral angles
of 0 and 180°. When $\theta = 90°$, induction from the adjoining electron deficient carbon
tightens the C-D bond, producing a small inverse isotope effect. The inductive effect
associated with deuterium isotope substitutions also contributes to the isotope effect, but
is seldom included in the analysis of the $\beta$-secondary isotope effects due to its small size.
Thus, β-secondary isotope effects can not only provide key information about the amount of positive charge formation on the reaction center at the transition-state, but they can also give pertinent information about the conformation of the reaction center due to the angular dependence of the isotope effect. The β-secondary deuterium isotope effects for traditional S_N2 and S_N1 reactions fall in the range of 1.00-1.02 and 1.08-1.15 per deuterium substitution, respectively.

**Kinetic Isotope Effect Measurement Technique**

Given that the majority of isotope effects are generally small with the exception of hydrogen primary isotope effects, the establishment of reliable methods that accurately and precisely measure the isotope effect is quite important in order to acquire the most information about the reaction mechanism. Depending on the type of method used to measure the isotope effect, one can determine whether the effect is on V/K or V_max. The two commonly used methods to measure kinetic isotope effects on enzyme catalyzed reactions are the competitive and non-competitive methods. These methods will be discussed in greater detail below.

**The Competitive Method**

The competitive method uses a mixture of two isotopically labeled substrates in a reaction with an enzyme. The reaction rates are then simultaneously measured for the two isotopically labeled substrates based on changes in the light/heavy isotope ratio over the time course of the reaction. In the competitive method, only V/K effects can be measured because the two isotopically labeled substrates are considered to be competitive inhibitors of each other and, therefore, both can never be saturating. The two most commonly used labels for the substrate isotopomer pair are ^3_H and ^14_C radiolabels. In this case, one substrate contains a radiolabel at the isotopically sensitive position, while
the other substrate contains a radionuclide at a remote position. In addition to these labels, stable isotopes such as $^{18}$O, $^{15}$N, and $^2$H, can also be used to label the substrate. In this situation, a dual-labeled substrate is synthesized that contains a stable isotope in the isotopically sensitive position and a radioactive trace label in a remote position. The competitive method is advantageous to use because the experimental errors are generally small, $<1\%$, even when unstable substrates are used.$^{11}$

Gas-ratio mass spectroscopy and dual-channel liquid scintillation counting are the two common techniques used to measure competitive method KIEs. The gas-ratio mass spectroscopy method can be advantageous to use because the experimental error is generally around 0.001%.$^{11}$ The major caveat to using this method is that it requires the isotopically labeled substrate to be converted into a gas. Additionally, this conversion must take place without an isotope effect occurring.$^{11}$ In this method, substrates containing stable isotope labels are generally preferred over those containing radionuclide labels because these substrates are more difficult to synthesize in large quantities and the radioactive precursors are often more expensive to purchase.

Dual-channel liquid scintillation counting is often chosen over gas-ratio mass spectroscopy to measure KIEs because it easier to use for substrates containing $^3$H and $^{14}$C radionuclide labels. The advantages of this technique are that it can clearly differentiate between the two radioisotopomers, it is highly sensitive, and it can easily be used for substrate radioisotopomers that have different specific activities without affecting the KIE results. One of the drawbacks to using this technique is a lower degree of precision than for gas-ratio mass spectroscopy methods, but the attainable value of $\pm 0.25\%$ is often sufficient for the problem to which it is applied. Furthermore, the synthesis of the
various substrate isotopomers still remains an obstacle since it is often very difficult to synthesize substrates with a radioisotope or a stable isotope label in the desired positions. The synthesis and purification of the desired substrates is usually “rate limiting step” toward obtaining KIE measurements. Synthetic routes that employ enzymes to synthesize many of the desired substrate isotopomers has grown in popularity in recent years because enzymes are highly stereospecific and selective. Additionally, enzymes are able to rapidly produce high yields of the desired product often in a convenient one-pot reaction mixture. The pitfalls of using enzymatic synthesis over traditional chemical synthesis are that the synthesis is limited by the types of reactions that enzymes can perform, and that many of the enzymes required are not commercially available.

When using the dual-channel liquid scintillation technique to measure KIE values, sample vials should be counted for at least 10 minutes each for a minimum of 6 cycles in order to reduce the relative standard deviation. The standard deviation for this technique approximately equals the square root of the number of counts, \( s = \text{cpm}^{(1/2)} \), for a single determination of counts. For example, the percent relative deviation for a sample containing at least 350,000 cpm is 0.3 %.\(^{11}\) KIE experiments should have a percent relative standard deviation of \(<0.25 \%\) for this method.\(^{11}\) The calculation of the KIEs using the dual-channel liquid scintillation method will be discussed in greater detail in the experimental section.

As with any experimental method, the establishment of several controls that test the validity of the method is paramount. When using dual-labeled substrates, one control that should be performed is the one that will demonstrate that the site of the remote radioactive trace label is in an isotopically insensitive position. This control is usually
done by measuring the KIE for the substrate isotopomers that only contain the radiolabel in the remote position. If a KIE is observed at this remote position, than this value can be used to correct future KIEs measured on dual-labeled substrates with these remote labels.

The measurement of KIEs using the dual-labeled competitive method typically employs the use of column chromatography (FPLC or HPLC) to separate reaction products from the remaining substrate. Therefore, another type of control that should be performed is the one that will test whether there is any isotopic fractionation from the column chromatography method. This control is typically accomplished by measuring the $^3\text{H}/^{14}\text{C}$ ratios of a substrate mixture prior to and after chromatography. Fractions are collected after the chromatography step into liquid scintillation vials for counting using a dual-channel liquid scintillation counter. Special care should be taken to collect the entire radioactive peak in order to ensure that there is an identical $^3\text{H}/^{14}\text{C}$ composition in all fraction vials for multiple trials. Identical $^3\text{H}/^{14}\text{C}$ ratios indicate that there is no isotopic fractionation from the column chromatography methods.

The Noncompetitive Method

In the noncompetitive method or “direct rate method”, the reaction rates of two isotopically labeled substrates are measured individually using separate enzyme reaction mixtures. Unlike the competitive method, this method can measure both V/K and $V_{\text{max}}$ values for the overall reaction or for a single turnover. The use of the noncompetitive method to measure small KIEs values is often undesirable because of the large experimental error (2-10 %) associated with this method. Thus, primary deuterium KIEs are typically the only KIEs measured with this method because their large KIE values are generally less affected by the magnitude of the error. However, errors of less than 1 % have been obtained for small heavy atom $^{15}\text{N}$ and $^{18}\text{O}$ kinetic isotope effects.
when special spectrophotometric techniques were used. Another caveat of this method is that it is difficult to exactly reproduce the experimental conditions for every reaction. Changes in substrate and enzyme concentrations, specific activities, and reaction temperature may give variable KIE values. Time-point assays are not a suitable method to use for the measurement of noncompetitive KIEs because of they can have errors greater than 10%. Therefore, noncompetitive KIEs are usually measured on a UV-vis spectrophotometer using the continuous assay method.

**Kinetic Isotope Effect Methodology**

The KIE methodology used in the following experiments was the dual-label competitive method. KIE measurements were made by incubating recombinant sialyltransferase with a mixture of dual-labeled CMP-NeuAc or UMP-NeuAc radioisotopomers and acceptor substrate until a substrate to product conversion of 40-60% was achieved. Unreacted substrate from a non-enzyme initiated reaction (t₀) and an enzyme initiated reaction (t₁/₂) were isolated by anion-exchange HPLC and directly collected into liquid scintillation vials for dual-channel liquid scintillation counting. The typical t₀ and t₁/₂ UMP-NeuAc HPLC chromatograms from a KIE experiment are shown in Figure 4-5.

UMP-NeuAc isotopomers were primarily used as the donor substrate for KIE experiments with the recombinant sialyltransferases. This was done in order to avoid the commitment factor associated with the natural donor substrate, CMP-NeuAc. As discussed previously in Chapter 2, previous work conducted in our laboratory showed that there is a commitment to catalysis (commitment factor, Cᵢ) for the CMP-NeuAc donor substrate when bound to sialyltransferase. This means that the sialyltransferase catalyzed reaction with CMP-NeuAc donor substrate contains more than one kinetic
barrier that is partially rate limiting.\textsuperscript{17} When a commitment factor exists, the non-chemistry rate limiting steps can mask the full expression of the KIEs. As a result, the observed KIE values are typically smaller than the actual (intrinsic) KIEs. The observed V/K isotope effects can be corrected for the commitment factors using equation 4-4.

\[
\text{KIE}_{\text{intrinsic}} = \text{KIE}_{\text{observed}} + (\text{KIE}_{\text{observed}} \times C_f) - C_f
\]  

eq. 4-4

One method to eliminate the commitment factor is to alter the reaction conditions in a way that will reduce the rate of the isotopically sensitive step. In our case, UMP-NeuAc removes the commitment factor in two ways. First, by raising the kinetic barrier for the chemistry step as indicated by its lower \( k_{\text{cat}} \) value (\( k_{\text{cat}} = 1.2 \text{ s}^{-1} \) for UMP-NeuAc and \( k_{\text{cat}} = 3 \text{ s}^{-1} \) for CMP-NeuAc).\textsuperscript{16} Secondly, the \( K_m \) for UMP-NeuAc is higher (\( K_m \text{ UMP-NeuAc} = 1.2 \text{ mM} \) vs. \( K_m \text{ CMP-NeuAc} = 16 \text{ µM} \)).\textsuperscript{16} UMP-NeuAc is also an ideal substrate for these isotope effect studies because the structure is similar to that of the natural donor substrate, CMP-NeuAc. This substrate only differs from CMP-NeuAc by one substitution, an amino group substituted for a keto group at the C4 position of the pyrimidine ring, which is distant from the anomeric carbon reaction center of NeuAc.
Figure 4-5. Typical $t_0$ (top panel) and $t_{1/2}$ (lower panel) UMP-NeuAc HPLC chromatograms for KIE experiments on recombinant sialyltransferase. The vertical lines represent the beginning and end of fraction collection.
Results and Discussion

The kinetic isotope effects measured for recombinant human α(2→3) sialyltransferase (Ins-h23STGal IV), rat α(2→3) sialyltransferase (r23STGal IV), and rat α(2→6) sialyltransferase (ST6Gal I) are listed in Tables 4-1 – 4-3, respectively.

Table 4-1. KIEs measured for recombinant human α(2→3) sialyltransferase (Ins-h23STGal IV).

<table>
<thead>
<tr>
<th>Isotopomeric Pair</th>
<th>Type of KIE</th>
<th>KIE Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-3H-NAc], [1-14C-NAc] CMP-NeuAc</td>
<td>Control</td>
<td>1.000 ± 0.005</td>
</tr>
<tr>
<td>[1-3H-NAc; 3,3'-2H2], [1-14C-NAc] UMP-NeuAc</td>
<td>β-Secondary</td>
<td>1.161 ± 0.010</td>
</tr>
<tr>
<td>[9-3H], [1-14C-NAc] CMP-NeuAc</td>
<td>Binding</td>
<td>0.963 ± 0.006</td>
</tr>
<tr>
<td>[9-3H], [1-14C-NAc] UMP-NeuAc</td>
<td>Binding</td>
<td>0.922 ± 0.010</td>
</tr>
</tbody>
</table>

Table 4-2. KIEs measured for recombinant rat α(2→3) sialyltransferase (r23STGal IV). The asterisk denotes the KIE previously measured by Michael Bruner.18

<table>
<thead>
<tr>
<th>Isotopomeric Pair</th>
<th>Type of KIE</th>
<th>KIE Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-3H-NAc], [1-14C-NAc] UMP-NeuAc</td>
<td>Control</td>
<td>0.988 ± 0.008</td>
</tr>
<tr>
<td>[9-3H], [1-14C-NAc] UMP-NeuAc</td>
<td>Binding</td>
<td>0.910 ± 0.006</td>
</tr>
<tr>
<td>[1-3H-NAc; 3,3'-2H2], [1-14C-NAc] UMP-NeuAc</td>
<td>β-Secondary</td>
<td>1.160 ± 0.010*</td>
</tr>
<tr>
<td>[1-3H-NAc], [1-14C-NAc, P18O2] UMP-NeuAc</td>
<td>Secondary 18O</td>
<td>0.981 ± 0.011</td>
</tr>
<tr>
<td>[1-3H-NAc], [1-14C-NAc, 2-18O] UMP-NeuAc</td>
<td>Primary 18O</td>
<td>1.018 ± 0.005*</td>
</tr>
<tr>
<td>[9-3H], [1-14C-NAc, 2-18O] UMP-NeuAc</td>
<td>Primary 18O</td>
<td>1.020 ± 0.003</td>
</tr>
</tbody>
</table>

Table 4-3. KIEs measured for recombinant rat α(2→6) sialyltransferase (ST6Gal I). Asterisks denote KIEs previously measured by Michael Bruner.18,19

<table>
<thead>
<tr>
<th>Isotopomeric Pair</th>
<th>Type of KIE</th>
<th>KIE Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-3H-NAc], [1-14C-NAc, P18O2] UMP-NeuAc</td>
<td>Secondary 18O</td>
<td>0.998 ± 0.008</td>
</tr>
<tr>
<td>[1-3H-NAc], [1-14C-NAc, 2-18O] UMP-NeuAc</td>
<td>Primary 18O</td>
<td>1.034 ± 0.007*</td>
</tr>
</tbody>
</table>

A KIE of 1.161 ± 0.010 was measured for recombinant human α(2→3) sialyltransferase (Ins-h23STGal IV) using the isotopomeric pair of [1-3H-NAc; 3,3'-2H2]
and [1-\textsuperscript{14}C-NAc] UMP-NeuAc in the presence of 300 mM α-lactose. This KIE value is characteristic of an S\textsubscript{N}1-like reaction mechanism because it is dissociative at the transition state without nucleophilic participation (S\textsubscript{N}1-like). This value was also identical within experimental error to the value previously measured by Michael Bruner for rat α(2→3) sialyltransferase (r23STGal IV).\textsuperscript{19} These KIE values were obtained after correcting for the small inverse KIE (0.988 ± 0.008) that was observed at the remote [1-\textsuperscript{3}H-NAc] label. The β-secondary dideuterium KIE results obtained for recombinant Ins-h23STGal IV and r23STGal IV are interesting because they are significantly smaller than the 1.218 ± 0.010 β-secondary KIE reported for recombinant r26STGal I using the same donor substrate isotopomeric pair.\textsuperscript{16} The smaller β-secondary dideuterium KIE values suggests that there is less positive charge at the reaction center on the NeuAc ring for the recombinant Ins-h23STGal IV and r23STGal IV transition-states. Furthermore, because there is less hyperconjugation occurring between the C3 NeuAc ring deuterons and the oxocarbenium ion in the transition-state, this suggests that the conformation of the NeuAc ring may be different for the recombinant α(2→3) sialyltransferase enzymes in the transition-state, compared to the ST6Gal I enzyme.

In addition to the β-secondary dideuterium KIEs, similar KIEs were also observed for the recombinant Ins-h23STGal IV and r23STGal IV enzymes at the C9 position on the NeuAc glycerol tail. Binding KIEs of 0.922 ± 0.010 and 0.910 ± 0.006 were measured for recombinant Ins-h23STGal IV and r23STGal IV, respectively, using the isotopomeric pair of [9-\textsuperscript{3}H] and [1-\textsuperscript{14}C-NAc] UMP-NeuAc in the presence of 300 mM α-lactose. The large inverse isotope effect observed at the C9 position on the donor substrate indicates that the bond to the tritium label becomes tighter in the transition-state.
when enzyme bound. This result would occur if binding interactions existed between the NeuAc glycerol tail hydroxy moieties and amino acids within the enzyme active-site.

The binding KIEs with UMP-NeuAc are larger than the effect observed at this position for recombinant Ins-h23STGal IV with the natural substrate CMP-NeuAc (0.963 ± 0.006). This result indicates that the majority of the commitments associated with CMP-NeuAc in the sialyltransferase catalyzed reaction have been removed and the binding KIEs measured for recombinant Ins-h23STGal IV and r23STGal IV with UMP-NeuAc are truly intrinsic. Furthermore, the binding KIE values for recombinant Ins-h23STGal IV and r23STGal IV were slightly larger than the 0.944 ± 0.010 binding KIE reported for recombinant ST6Gal I using the same donor substrate isotopomeric pair.16 This result suggests that the binding interactions between the NeuAc glycerol tail and enzyme active-site amino acid residues may be different for the recombinant ST6Gal I enzyme in the transition-state. One way this would occur would be if recombinant ST6Gal I has different active-site amino acid residues interacting with the hydroxy moiety of the glycerol tail.

Despite this difference, the binding KIE results for the recombinant Ins-h23STGal IV, r23STGal IV and ST6Gal I enzymes may be useful in the area of sialyltransferase inhibitor design. The binding KIE results suggest that this segment of the donor substrate experiences a binding interaction with the enzyme. Thus, the glycerol tail may be an important component to consider when designing new sialyltransferase inhibitors. Many of the reported sialyltransferase inhibitors maintain this portion of the donor substrate.20-22 However, some of the most potent sialyltransferase inhibitors contain a phenyl group instead of the glycerol tail on the NeuAc ring.21,23 These results suggest that
sialyltransferases are capable of tolerating a variety of modifications made to this part of
the donor substrate by utilizing compensatory binding interactions.

Since one of the primary goals of this project is to probe the mechanism of glycosyl
transfer with respect to interactions at the phosphate leaving group, several primary and
secondary leaving group $^{18}$O KIEs were measured for recombinant r23STGal IV and
r26STGal I enzymes. As mentioned in Chapter 1, the profile from the pH-rate
experiments on recombinant r26STGal I using UMP-NeuAc and LacNAc as the donor-
acceptor substrate pair fits a bell-shaped curve for two ionizable groups with pKa values
of 6.2 and 8.9.16 These data suggest that glycosyltransfer proceeds via a general acid
catalyzed mechanism in which one of the phosphate oxygens on the donor substrate may
be protonated to facilitate the loss of the nucleotide monophosphate moiety. Thus, the
results from the leaving group $^{18}$O KIE experiments may be useful in pinpointing the
location of the proton at either the bridging or non-bridging phosphate oxygens of the
donor substrate.

Before discussing the results of the leaving group $^{18}$O KIE experiments, it is
important to mention that results from positional isotope exchange (PIX) experiments
with CMP-NeuAc indicated that the phosphate group of the leaving CMP moiety did not
rotate and re-establish the bond with the NeuAc oxocarbenium ion prior to it departing as
shown in Figure 4-6.19 This aspect of the mechanism is important to consider since the
following leaving group $^{18}$O KIEs were measured using donor substrates containing
specific phosphate $^{18}$O labels. Rotation of the phosphate group would have adversely
affected the measurement and interpretation of the leaving group $^{18}$O KIEs because the
isotopically labeled positions on the phosphate group of the donor substrates would no
longer be static. Indeed, the results obtained in this work (Tables 4-2, 4-3) for primary vs. secondary $^{18}$O KIEs argue against any significant PIX, since one would have anticipated identical KIEs if pixing had occurred to any significant extent.

**Positional Isotope Exchange (PIX)**

![Positional Isotope Exchange (PIX) diagram]

R = Cytidine

![Positional Isotope Exchange (PIX) diagram]

Figure 4-6. Positional isotope exchange (PIX) mechanism. PIX could not be detected for the sialyltransferase mechanism. If pixing is complete, the bridge $^{18}$O label scrambles to give a 33 % $^{18}$O distribution at each oxygen.

Secondary $^{18}$O KIEs of 0.981 ± 0.011 and 0.998 ± 0.008 were measured for recombinant r23STGal IV and r26STGal I enzymes, respectively, using [$1^{-3}$H-NAc] and [$1^{-14}$C-NAc, $^{18}$O$_2$] UMP-NeuAc as the donor substrate isotopomeric pair. Additionally, primary $^{18}$O KIEs of 1.018 ± 0.005 and 1.034 ± 0.007 were observed for recombinant
r23STGal IV and r26STGal I enzymes, respectively, using [1-3H-NAc] and [1-14C-NAc, 2-18O] UMP-NeuAc as the donor substrate isotopomeric pair. The primary 18O KIE experiment for recombinant r23STGal IV was repeated using [9-3H] and [1-14C-NAc, 2-18O] UMP-NeuAc as the donor substrate isotopomeric pair in order to confirm the results obtained by Bruner. This experiment resulted in an observed isotope effect of 1.020 ± 0.003 after correcting the KIE for the isotope effect at the remote 9-3H label. This result was virtually identical to the primary 18O KIE measured by Bruner with this enzyme.

In order to determine the meaning of these leaving group 18O KIEs, a series of theoretical calculations were done to predict the KIEs using the change in bond order for each protonation case (non-bridging, bridging, and no protonation) in going from the ground-state to the transition-state based on a maximum 18O EIE of 1.06 for a loss of a complete bond. Protonation of one of the non-bridging phosphate oxygens resulted in an increase in the bond order of 0.25 for the [P18O2] CMP-NeuAc model and a decrease in the bond order of 0.5 for the [2-18O] CMP-NeuAc model as shown in Figure 4-7. This protonation case resulted in predicted primary and secondary 18O KIEs of ~1.02 and 0.98 – 1.00 for the [2-18O] CMP-NeuAc and [P18O2] CMP-NeuAc models, respectively (Table 4-4). Furthermore, protonation of the bridging phosphate oxygen resulted in predicted primary and secondary 18O KIEs of unity since the change in bond order was zero for both of the bridging and nonbridging 18O labeled CMP-NeuAc models (Figure 4-7 and Table 4-4). Finally, the no protonation case resulted in a decrease in bond order of 0.67 for the [2-18O] CMP-NeuAc model and a decrease in bond order of 0.17 for the [P18O2] CMP-NeuAc model (Figure 4-8). These results gave predicted primary and secondary
$^{18}$O KIEs of >1.02 and ~1.01 for the bridging and non-bridging $^{18}$O labeled substrate models, respectively.

Table 4-4. Summary of predicted KIEs based on mechanism.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Bridge $^{18}$O KIE</th>
<th>Non-bridge P$^{18}$O$_2$ KIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-bridge Protonation</td>
<td>~1.02</td>
<td>0.98 – 1.00</td>
</tr>
<tr>
<td>Bridge Protonation</td>
<td>~1.00</td>
<td>~1.00</td>
</tr>
<tr>
<td>No Protonation</td>
<td>&gt;1.02</td>
<td>~1.01</td>
</tr>
</tbody>
</table>

**Non-bridge Protonation**

Figure 4-7. Bond order analysis for protonation at the non-bridging phosphate oxygen of the donor substrate.
Figure 4-8. Bond order analysis for protonation at the bridging phosphate oxygen of the donor substrate (top panel) and no protonation of the phosphate oxygens of the donor substrate (lower panel).
When compared to the models, the observed leaving group $^{18}$O KIEs agreed with the predicted $^{18}$O KIE values for a mechanism involving proton transfer at the non-bridging phosphate oxygen. The small inverse secondary $^{18}$O isotope effects measured on recombinant r23STGal IV and r26STGal I support a dissociative type mechanism in which the P-O bond order increases in the transition-state as shown in the model for non-bridge protonation (Figure 4-7).$^{25,26}$ Although it is not obvious why acid catalysis would be necessary to assist in the departure of a stable leaving group, the anionic non-bridging phosphate oxygen would be a reasonable location for a proton since this oxygen should be more basic than the bridging phosphate oxygen at the transition-state. On the other hand, the bridging phosphate oxygen would also appear to be a reasonable location for a proton since protonation here would weaken the glycosidic bond by making the phosphoryl group more electrophilic and possibly expedite the rate of glycosyl transfer.$^{16}$ Protonation of the bridging phosphate oxygen in this case is extremely thermodynamically unfavorable given the low basicity of this phosphate oxygen. Glycosides, such as lysozyme, that use acid catalysis must protonate at the bridging oxygen because this is the only suitable location for protonation to occur on the donor substrate.$^{27,28}$ However, glycosyltransferases use donor substrates that contain at least two non-bridging phosphate oxygens which are significantly more basic than the bridging oxygen. Protonation of these non-bridging phosphate oxygens would still render the nucleotide monophosphate a better leaving group. Thus, the results suggest that glycosyl transfer proceeds via a general acid catalyzed mechanism in which a non-bridging phosphate oxygen is protonated to facilitate the loss of CMP.
In addition to the secondary $^{18}\text{O} \text{KIE}$ results, the magnitudes of the primary $^{18}\text{O}$ KIEs indicate that there is significant bond cleavage to the leaving group in the transition-state. The observation that the primary $^{18}\text{O}$ KIEs measured for recombinant r23STGal IV are slightly smaller than the primary $^{18}\text{O}$ KIE measured for recombinant r26STGal I suggests that there is less bond cleavage to the leaving group for the r23STGal IV enzyme in the transition-state. This could be due to the reaction proceeding with an earlier transition-state. The $\beta$-secondary dideuterium KIEs previously measured for the two recombinant enzymes also suggest that there is less glycosidic bond cleavage in transition-state for recombinant r23STGal IV, so the results of both $^2\text{H}$ and $^{18}\text{O}$ KIEs are in agreement. Thus, two different transition-states can be proposed for the recombinant r26STGal I and r23STGal IV enzymes based on the KIE results as shown in Figure 4-9. Furthermore, it is also likely that the transition-state for recombinant Ins-h23STGal IV will be similar to the transition-state proposed for recombinant r23STGal IV given that the secondary $\beta$-dideuterium and the 9-$^3\text{H}$ binding KIEs were similar for these two enzymes.

Figure 4-9. Transition-state models proposed for recombinant r26STGal I (left) and r23STGal IV (right) enzymes.
Conclusions

The library of isotope effects measured for the recombinant human $\alpha(2\rightarrow3)$, rat $\alpha(2\rightarrow3)$, and rat $\alpha(2\rightarrow6)$ sialyltransferases support a dissociative, $S_N1$-like transition-state with substantial oxocarbenium ion character. The results from the leaving group $^{18}\text{O}$ KIE studies on recombinant rat $\alpha(2\rightarrow3)$, and rat $\alpha(2\rightarrow6)$ sialyltransferases suggest that the sialyltransferase catalyzed mechanism proceeds via acid catalysis on the non-bridging phosphate oxygen. Furthermore, the secondary $\beta$-dideuterium and primary $^{18}\text{O}$ leaving group effects for the recombinant rat $\alpha(2\rightarrow3)$ enzyme indicate that there is less charge build-up and C-O bond cleavage for this enzyme than the rat $\alpha(2\rightarrow6)$ sialyltransferase enzyme in the transition-state. These effects suggest that donor substrate structures for the recombinant rat $\alpha(2\rightarrow3)$ and rat $\alpha(2\rightarrow6)$ sialyltransferase catalyzed mechanisms are slightly different in the transition-state. This knowledge about the behavior of the transition-states for these two enzymes is important in the area of sialyltransferase inhibitor design because it may lead to the creation of new sialyltransferase transition-state inhibitors that are specific to each enzyme. Transition-state analogs that mimic an early transition-state may be more specific to the recombinant $\alpha(2\rightarrow3)$ sialyltransferases, while those that mimic a late transition-state may better suited for recombinant $\alpha(2\rightarrow6)$ sialyltransferase inhibition. Evidence to support this inhibitor design strategy can been seen in the work conducted by Vern L. Schramm where early and late transition-state analogs were used to target the inhibition of purine nucleoside phosphorylase (PNP) from *M. tuberculosis* (Figure 4-10).
Figure 4-10. Early and late transition state analogs for PNP from Schramm.  

**Experimental**

**Enzyme Reaction General KIE Methodology**

The competitive method was employed to measure the kinetic isotope effects (KIEs) for recombinant human α (2→3), rat α (2→3), and rat α (2→6) sialyltransferases. For each different KIE experiment, a master mixture of the appropriate $^3$H- and $^{14}$C-labeled donor substrate was prepared from which aliquots were withdrawn to make the desired $^3$H/$^{14}$C reference mixture at time zero and the individual reaction mixtures. The reaction mixtures contained about 100,000 cpm of the appropriate $^3$H/$^{14}$C-labeled isotopomeric pair of CMP-NeuAc or UMP-NeuAc donor substrate and 300 mM α-lactose acceptor substrate in 50 mM MES, pH 7.2 containing 0.2 mg/mL BSA and 0.05 % (v/v) Triton CF-54 for the recombinant human and rat α (2→3) sialyltransferase KIEs. KIEs measured on recombinant rat α (2→6) sialyltransferase used 10 mM N-acetyl
lactosamine (LacNAc) as the acceptor substrate in 50 mM MES, pH 7.0 containing 1 mg/mL BSA, and 0.05 % (v/v) Triton CF-54. Reaction mixtures (20–100 µL) were initiated by the addition of the appropriate amount of enzyme to give 40-60 % conversion in < 30 minutes at 37 °C. Unreacted substrate was then isolated from the reaction mixture using anion-exchange HPLC (MonoQ column, 75 mM NH₄HCO₃, 15 % methanol, pH 8.0, 2 mL/min, A₂₇₁) and 2 mL fractions were directly collected into scintillation vials. Special care was taken to collect the entire CMP-NeuAc or UMP-NeuAc peak. The reaction conversion was determined by a peak integration of the CMP-NeuAc or UMP-NeuAc and CMP or UMP peaks in the HPLC chromatogram. The reference ³H/¹⁴C ratio at time zero was obtained for each enzyme reaction mixture by injecting aliquots of the appropriate ³H/¹⁴C labeled donor substrate mixture with acceptor substrate in buffer onto the anion-exchange column and collecting the entire donor substrate peak into liquid scintillation vials.

Dual channel liquid scintillation counting was used to determine the ³H/¹⁴C ratios for the donor substrates (channel A, 0-15 keV; channel B, 15-90 keV). Each tube was counted for 8-10 minutes and all tubes were cycled through the counter 8-10 times. Triplicate samples of [¹⁴C] CMP-NeuAc were used to determine the ratio of ¹⁴C counts in channels A and B (A:B₁₄). Since ³H is only detected in channel A, the ³H/¹⁴C ratio in a given sample tube was calculated with the following equation:

\[
³H/¹⁴C = \frac{\text{cpm} A - \text{cpm} B \times A:B_{14}}{\text{cpm} B + \text{cpm} B \times A:B_{14}}
\]

eq. 4-5
The reported value and error of a KIE represents the mean and standard deviation of 5-6 individual KIE experiments taken over 8-10 cycles through the liquid scintillation counter. The following equation was used to calculate the KIEs:

\[ ^{3}H \text{ KIE}_{\text{observed}} = \left( \frac{^{3}H/{^{14}}C}{^{3}H/{^{14}}C} \right)_{t_{1/2}} / \left( \frac{^{3}H/{^{14}}C}{^{3}H/{^{14}}C} \right)_{t_{0}} \]  
\[ \text{eq. 4-6} \]

\[ ^{14}C \text{ KIE}_{\text{observed}} = \left( \frac{^{14}C/^{3}H}{^{14}C/^{3}H} \right)_{t_{1/2}} / \left( \frac{^{14}C/^{3}H}{^{14}C/^{3}H} \right)_{t_{0}} \]  
\[ \text{eq. 4-7} \]

The KIEs were then corrected for percent conversion using the following equation \(^{33}\):

\[ \text{KIE}_{\text{corrected}} = \frac{\ln(1-f)}{\ln((1-f) \times \text{KIE}_{\text{observed}})} \]  
\[ \text{eq. 4-8} \]

\[ f = \text{fraction of conversion} \]

For KIEs involving stable isotopes where complete incorporation was not achieved (\(^{18}O\) KIEs), the following equation was used to correct the observed KIEs \(^{34}\):

\[ \text{KIE}_{\text{corrected}} = \frac{(\text{KIE}_{\text{observed}} - 1 + f)}{f} \]  
\[ \text{eq. 4-9} \]

\[ f = \text{fraction of stable isotope incorporation} \]

Additionally, the following equations were used to correct the KIE if a KIE was observed at the remote position on the dual-labeled substrates\(^{11}\):

\[ \text{Corrected KIE for}^{14}C \text{ KIE} = \text{KIE}_{\text{observed}} \times \text{control KIE} \]  
\[ \text{eq. 4-10} \]
Corrected KIE for $^3$H KIE = $\frac{KIE_{observed}}{control}$ KIE  

\text{eq. 4-11}
CHAPTER 5
CONCLUSIONS AND FUTURE WORK

This study has provided an increased understanding of the transition-state and the mechanism of sialyl transfer for the reactions catalyzed by the recombinant human α(2→3), rat α(2→3), and rat α(2→6) sialyltransferases. In addition to the mechanistic information, valuable knowledge has been gained pertaining to the purification and kinetic properties of three recombinant human α(2→3) sialyltransferase isoforms expressed from insect cells.

Kinetic isotope effect methods were used to obtain information pertaining to the transition-state structure of the sialyl donor. The series of isotope effects measured for the recombinant Ins-h23STGal IV, r23STGal IV, and ST6Gal I sialyltransferases support a dissociative, S_N1-like transition-state with substantial oxocarbenium ion character. Kinetic isotope effect methods were also used to probe the mechanism of glycosyl transfer with regard to enzyme interactions at the phosphate leaving group. Primary and secondary \(^{18}\)O leaving group isotope effects were measured on recombinant r23STGal I and ST6Gal I using a set of UMP-NeuAc isotopomers containing \(^{18}\)O labels either at the bridging or non-bridging phosphate oxygens. The results from the leaving group \(^{18}\)O KIE experiments on recombinant r23STGal IV and ST6Gal I, as well as the results from theoretical calculations, suggest that glycosyl transfer proceeds via a general acid catalyzed mechanism in which a non-bridging phosphate oxygen is protonated to facilitate the loss of CMP.
Additional KIE studies are now needed to ascertain whether acid catalysis occurs at either the proR or proS non-bridging phosphate oxygen of the donor substrate. These studies would require the synthesis of proR and proS phosphate $^{18}$O donor substrate isotopomers. Future work may involve the design and use of a new enzymatic synthesis route to selectively incorporate an $^{18}$O label into the proR or proS phosphate oxygen of the donor substrate.

The $\beta$-secondary dideuterium isotope effects observed for recombinant r23STGal I and h23STGal IV were smaller than those observed for recombinant ST6Gal I, suggesting that the degree of glycosidic bond cleavage is slightly different between the $\alpha(2\rightarrow3)$ and $\alpha(2\rightarrow6)$ sialyltransferases at the transition-state. These results in conjunction with the primary $^{18}$O leaving group isotope effect results suggest that the recombinant r23STGal I and h23STGal IV catalyzed reactions proceed with a slightly earlier transition-state than the recombinant ST6Gal I catalyzed reaction.

Thus, the information gleaned from this study may be useful in the area of sialyltransferase inhibitor design. Our results point towards a transition-state for $\alpha(2\rightarrow3)$ sialyltransferases that contains less positive charge and glycosidic bond cleavage than the transition-state for $\alpha(2\rightarrow6)$ sialyltransferase. Therefore, transition-state inhibitors that mimic the early and late transition-states of the $\alpha(2\rightarrow3)$ and $\alpha(2\rightarrow6)$ sialyltransferases would presumably make the inhibitors more specific to each enzyme. Furthermore, the most potent sialyltransferase inhibitors are the transition-state inhibitors that include a planar anomeric carbon, charge mimicry, the cytidine moiety, and an increased distance between the anomeric carbon and the leaving group CMP. These criteria are included in the proposed transition-state inhibitor shown in Figure 5-1 which is currently being
synthesized in our laboratory for future inhibition studies on both α(2→3) and α(2→6) sialyltransferases.

Figure 5-1. Proposed sialyltransferase transition-state inhibitor.

In addition to the sialyltransferase inhibition studies, future investigations of the sialyltransferase catalyzed mechanism may involve the measurement of KIEs on the bacterial sialyltransferase, CstIIΔ32. Although bacterial sialyltransferases do not share sequence similarity with any mammalian sialyltransferases, KIE studies may allow for mechanistic comparisons to be made between these two seemingly unrelated groups of sialyltransferases. The crystal structure of CstIIΔ32 could then be used to model the transition-state structure of the sialyl donor in the enzyme active site. This information may provide new insight into the nature of the mammalian sialyltransferase catalyzed reactions.
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BIOGRAPHICAL SKETCH

Erin Elizabeth Burke, daughter of Mr. and Mrs. Paul M. Ringus, was born in Greenfield, Massachusetts, on October 14, 1978. Erin lived in Athol, Massachusetts, until the age of six and then her family relocated to Moosup, Connecticut, in 1985 where she lived until the age of twelve. In the Fall of 1990, Erin’s family relocated to Barnwell, South Carolina, where she completed her secondary education culminating in graduation from Barnwell High School in 1996. She attended Columbia College in Columbia, South Carolina, where she graduated summa cum laude and cum honorae in 2000 with a Bachelor of Science degree in chemistry. Erin relocated to Gainesville, Florida, to pursue her Doctor of Philosophy degree in chemistry from the University of Florida. While in graduate school, Erin met and married her husband, Andrew Paul Burke, on December 7, 2002. Erin received her Ph.D. in chemistry in the summer of 2005 under the guidance of Dr. Nicole A. Horenstein. After graduation, Erin and her husband will be relocating to Charleston, South Carolina, to begin their new jobs.