Development of Macromolecular Acceptors for the Sialyltransferase ST6GlcNAcI from Bovine Glycoproteins

By

Erin Marie Szidonya

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Chemistry
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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TABLE OF CONTENTS

ABBREVIATIONS

Carbohydrate Subunits

NeuAc	*N*-acetyl-D-neuraminic acid (sialic acid)
Man	D-mannose
Glc	D-glucose
GlcNAc	D-*N*-acetylglucosamine
Gal	D-galactose
GalNAc	*N*-acetylgalactosamine
Fuc	Fucose

Glycosyltransferases

General

GT	Glycosyltransferase
ST	Sialyltransferase
GlcT	Glucosyltransferase
FucT	Fucosyltransferase
GalT	Galactosyltransferase
ManT	Mannosyltransferase
GlcNAcT	*N*-acetylglucosaminyltransferase

Specific (Residue which binds new sialic acid residue in bold type)

GlcNAcT-I	*N*-acetylglucosaminyltransferase I
GlcNAcT-III	*N*-acetylglucosaminyltransferase III
OST	Oligosaccharyltransferase
GluT	UDP-Glc:glycoprotein glycosyltransferase
ST6GlcNAcI	NeuAcα2,3Galβ1,3**GlcNAc**β-R α2,6-sialyltransferase
ST3GalII	β-D-**Gal**β1,3GalNAcβ-R α2,3-sialyltransferase

ST3GalIII	β-D-**Gal**β1,3(4)GlcNAcβ-R α2,3-sialyltransferase
ST6GalI	β-D-**Gal**β1,4GlcNAcβ-R α2,6-sialyltransferase
SAT-I	β-D-**Gal**α1,4-D-Glc-β-1-O-Cer α2,3 sialyltransferase

Acceptor Designations

As (or +*G* for α_1-AGP)	Asialoglycoprotein
As-O (or +*G* for fetuin)	Asialoglycoprotein, no O-linked chains
-G/A	Asialoglycoprotein, no O-linked chains, no β1,4-Gal
S	α2,3-Sialylated glycoprotein, no O-linked chains, no β1,4-Gal
DS	Desialylated glycoprotein, no O-linked chains, no β1,4-Gal

Materials and Methods

MWCO	Molecular Weight Cutoff
Da	Dalton
KDa	Kilodalton
RPM	Revolutions per minute
g_{av}	Average gravitational force
TCA	Trichloroacetic Acid
PTA	Phosphotungstic Acid
dpm	disintegrations per minute
Δdpm	dpm (test sample) – dpm (no acceptor)
cpm	counts per minute
Δcpm	cpm (test sample) – cpm (no acceptor)

General Text

α_1-AGP	α_1-Acid Glycoprotein

EDTA	Ethylenediaminetetraacetic Acid
DNA	Deoxyribonucleic Acid
cDNA	Complementary Deoxyribonucleic Acid
CDG	Congenital Disorders of Glycosylation
Asn	Asparagine
Ser	Serine
Thr	Threonine
ER	Endoplasmic Reticulum
TGN	*Trans* Golgi Network
UDP-Glc	Uridine diphosphate D-glucose
UDP-GlcNAc	Uridine diphosphate *N*-acetyl-D-glucosamine
GDP-Man	Guanidine diphosphate D-Mannose
Dol-P-Man	Dolichol monophosphate D-Mannose
Dol-P-Glc	Dolichol monophosphate D-Glucose
NRD	Nucleotide Recognition Domain
CTS	Cytoplasmic Transmembrane Stem
G_{M1}	Ganglioside$_{M1}$; β-D-Galβ1,3GalNAcβ1,4Galβ1,4Glcβ-1-O-Cer
Cer	Ceramide
K_m	Michaelis constant
V_{max}	Maximum rate
Sialyl-Le^x	Sialyl-Lewis^x
Sialyl-Le^a	Sialyl-Lewis^a
LNT	Lacto-*N*-tetraose
LST_a	LS-tetrasaccharide$_a$
LST_b	LS-tetrasaccharide$_b$
DSL	Disialyllacto-*N*-tetraose
HPLC	High Performance Liquid Chromatography
NMR	Nuclear Magnetic Resonance
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
HPAEC-PAD	High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Rat, human, and bovine glycoproteins contain the structure NeuAcα2,6GlcNAc on N-linked carbohydrate chains (Townshend *et al.*, 1989). The enzyme that creates this linkage, ST6GlcNAcI, has a specificity for addition to the NeuAcα2,3Galβ1,3GlcNAc terminal trisaccharide of these chains (Paulson *et al.*, 1984). The enzyme activity has been studied using only oligosaccharide acceptors. The main focus of this thesis has been to prepare potential macromolecular glycoprotein acceptors to study this enzyme activity using bovine fetuin and α_1-AGP as starting materials. To remove O-linked chains, the glycoproteins were treated with sodium borohydride in the presence of cadmium acetate/EDTA which results in minimal damage to N-linked structures and the protein backbone while specifically cleaving O-linked structures (Likhosherstov *et al.*, 1990). NeuAc was removed by dilute acid hydrolysis and β1,4-Gal structures were removed by the action of β1,4-galactosidase from *Streptococcus pneumoniae*. NeuAc was then re-added to the N-linked β1,3-Gal positions by ST3GalIII to produce the correct acceptor structure. At each stage in the conversion to the final product the acceptors were checked against three purified ST's (ST6GalI and ST3GalIII for N-linked chains and ST3GalII for O-linked chains). The completed acceptors were tested for ST6GlcNAcI activity with rat liver Golgi as enzyme source and the kinetics were analyzed. Furthermore, glycan structural information was obtained on the poorly-characterized bovine α_1-AGP. Supported by NSERC, Canada.

INTRODUCTION

Chapter I: The Role of Glycosyltransferases in Protein Glycosylation

Without a doubt, many of the greatest scientific developments in the field of biochemistry have occurred throughout the 20th century. From the elucidation of the double helical structure of DNA in the 1950s (Watson and Crick, 1953) to the development of antibiotics to treat once-fatal bacterial diseases to tremendous strides in the understanding of protein structure and function, we now have a much greater understanding of biomolecules and the forces that govern them. However, one area of biochemistry that had eluded study until the latter part of the century is glycobiology, or the study of carbohydrate-containing compounds. Before this was considered a major area of study, it was assumed that, for example, the protein or lipid to which the carbohydrate structure is bound is of primary importance to the function of the complete structure, and the attached carbohydrate was given little consideration. However, eventually the thinking changed, and it has now been shown that carbohydrates do indeed have a great deal of important biological functions rivaling those of the biomolecules they are attached to. For instance, carbohydrates have been found to be involved in many important functions in eukaryotic organisms, such as inflammation, cell-cell recognition, acting as receptors for toxins or other macromolecules or as 'molecular contacts' between hosts and pathogens, determining blood group antigenic structures, and providing shock absorption in the joints in mammals in the form of proteoglycans, to name just a few (Gagneux and Varki, 1999). In fact, modification of proper carbohydrate structure has been found to result in certain serious diseases, such as congenital disorders of glycosylation (CDG, several types) or mucolipidosis II (I-cell disease) in humans.

Clearly, such a wide range of carbohydrate functions suggests the requirement for a wealth of varied carbohydrate structures. Consequently, in the past twenty-five years, there has been a tremendous amount of interest in studying glycan structures and the enzymes that create them, the glycosyltransferases (GT's), a large family of enzymes of shared ancestral origins. Contrary to the methods of synthesis of proteins and DNA, glycans are not made from templates; rather, their structures are determined by the substrate specificity of the various GT's that transfer nucleotide sugars onto a precursor glycan chain. As the aim of this thesis is primarily to create a protein-bound carbohydrate of the correct structure through the use of these enzymes, the following section will focus on features of the GT's with regards to their function in protein glycosylation, general reaction properties, localization and structural features, as well as discussing the production of recombinant forms of these enzymes.

A. Creating a Precursor: Preliminary Stages in Protein Glycosylation

Glycoproteins themselves can be divided into two distinct groups; the O-linked glycoproteins, in which the glycan structure is covalently bound to the oxygen atom in the R-group of a threonine or serine residue in the protein, and the N-linked glycoproteins, which have the carbohydrate attached to the R-group nitrogen of an asparagine residue (see Figure 1). In O-linked glycosylation, a monosaccharide (usually GalNAc) is attached directly to the hydroxyl group of either serine or threonine, with additional sugars being added on in a reasonably straightforward stepwise fashion in the Golgi apparatus by the GT's. On the other hand, the much better-characterized Asn-

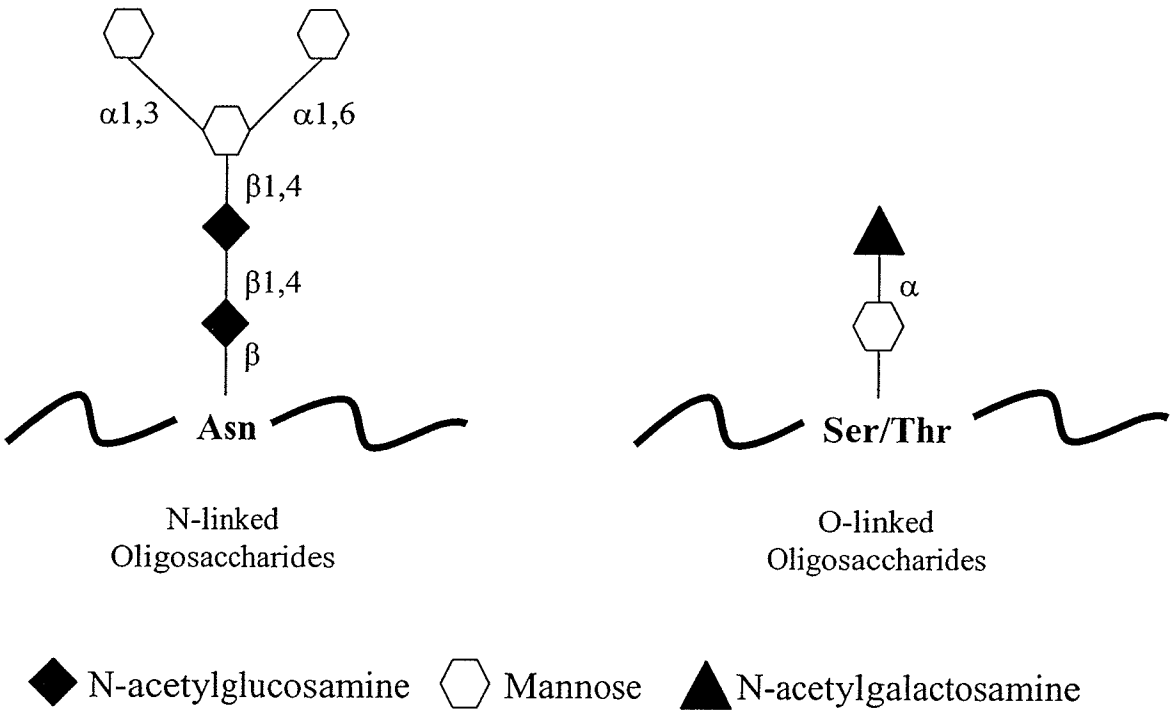

Figure 1. The basic core structures of protein-bound glycans. Included in this diagram are the linkages found between the individual monosaccharides in the Asn- or Ser/Thr-linked core structures, with the numbers representing the carbons of the monosaccharide involved in the bond, and the designation α or β referring to the conformation of the anomeric carbon (Diagram adapted from Varki, 1998).

linked glycosylation is significantly more complicated, involving the creation of a precursor structure before further carbohydrate extension in the Golgi can occur. This mode of synthesis will be the primary focus here. In N-linked glycoproteins, there is an initial transfer of cytosolically-located UDP-GlcNAc by the enzyme N-acetylglucosaminyl phosphate transferase onto a lipid intermediate known as dolichol phosphate which is embedded in the ER membrane. This constitutes the first step in what is commonly known as the 'dolichol phosphate cycle,' essentially the pathway by which Asn residues become glycosylated (see Figure 3) (Sharon and Lis, 1981; pathway reviewed in Burda and Aebi, 1999). The amphipathic dolichol phosphate is a polyisoprenoid containing 16-23 repeating isoprene units (with eukaryotic cells usually having 19) attached to an additional α-saturated isoprene unit and a phosphate group (Jung and Tanner, 1973; Schutzbach, 1997):

$$(CH_3)_2C{=}CH\text{-}CH_2\text{-}[CH_2\text{-}(CH_3)C{=}CH\text{-}CH_2]_{15\text{-}22}\text{-}CH_2\text{-}(CH_3)CH\text{-}(CH_2)_2\text{-}O\text{-}P\text{-}O_3^{-2}$$

Basically, dolichol phosphate acts as the 'carrier molecule' in the assembly of N-linked glycans, with a common carbohydrate structure first being constructed on a dolichol phosphate backbone in the ER. Only after this happens can the transfer of the carbohydrate structure onto the protein and further glycan modification occur. In precursor synthesis, the carbohydrates themselves are attached to dolichol phosphate by means of sequential addition of monosaccharide units as high-energy diphosphate or monophosphate nucleotides by GT's to the growing end of the glycan chain. There are generally six main types of these monosaccharides found in glycoproteins, each

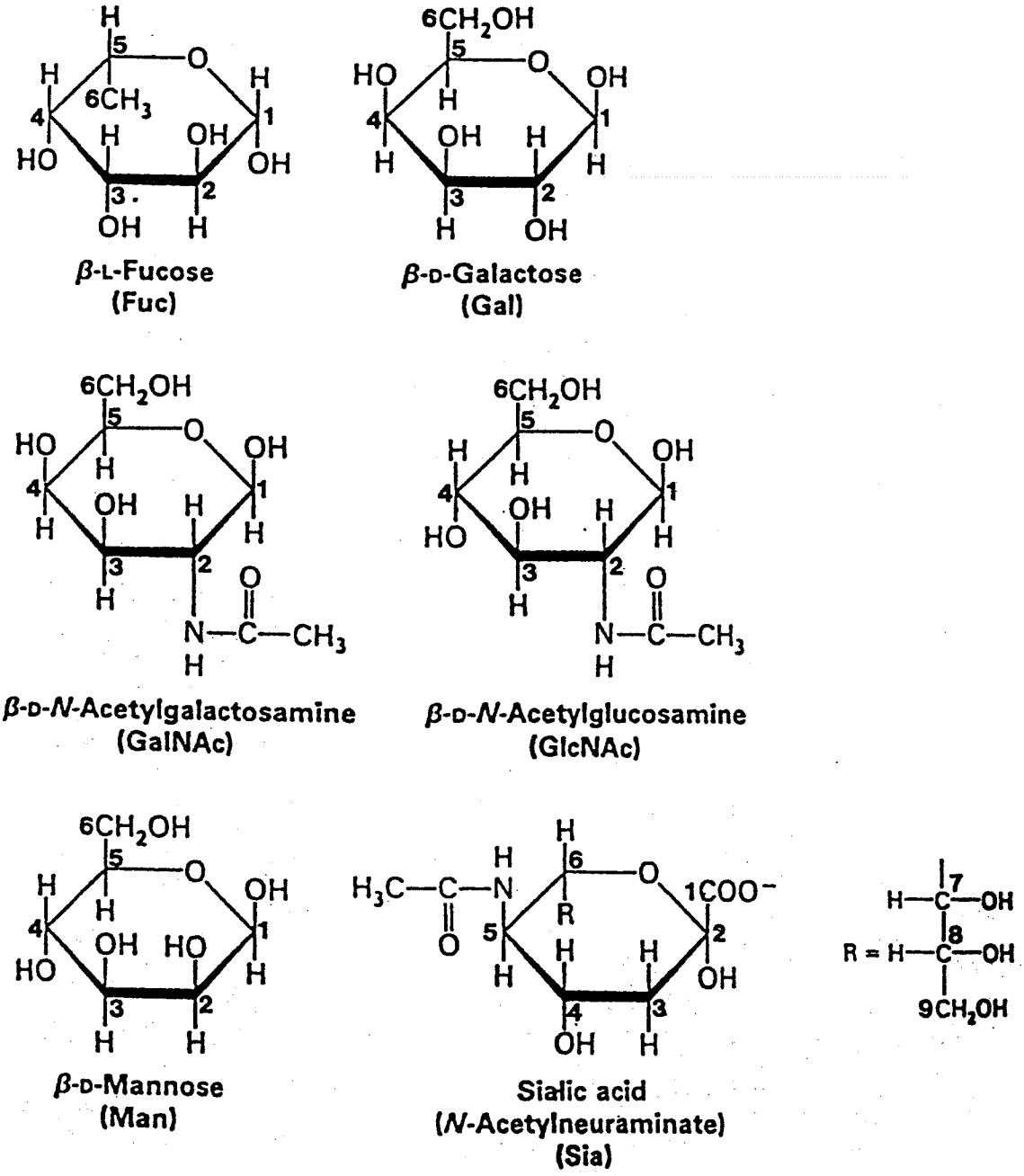

Figure 2. The monosaccharides utilized in the synthesis of protein-bound glycans. The carbon present at position 1 (position 2 in NeuAc) is referred to as the 'anomeric' carbon. Hydroxyl groups present above the plane of the ring are said to be in β-configuration, or α-configuration when below, with fucose being the exception to this rule as it is an L-sugar while all the others are D-sugars. The linkage types created when two of these sugar residues are joined are named based on the carbons involved in the linkage. For instance, if the C-2 hydroxyl of NeuAc binds to the C-6 hydroxyl of Gal via a dehydration reaction, it would be termed an 'α2,6' linkage, whereas the reaction of the C-1 hydroxyl of Gal with the C-4 hydroxyl of GlcNAc would be a 'β1,4' linkage (diagram taken from Richardson, 1998).

composed of a six-carbon ring with hydroxyl and acetyl groups at varying positions (see Figure 2). The monosaccharides must first be 'charged' on a phosphate nucleotide before they can be added by the correct GT to the growing carbohydrate structure, and there are two pathways found in the eukaryotic cytosol for the synthesis of these compounds, with both utilizing negative feedback loops to prevent overproduction of a certain nucleotide sugar. Moreover, GDP-Man and UDP-Glc must be charged on dolichol phosphate themselves as Dol-P-Man and Dol-P-Glc before they can be added to the chain during these preliminary steps, an additional step which also occurs in the cytoplasm. During precursor synthesis, the hydrophobic lipid portion of the dolichol phosphate associates with the lipid membrane of the ER, allowing the polar phosphate group to project out into the cytosol and come into contact with the monosaccharide substrates.

Following the addition of the first GlcNAc residue to the phosphate end of the lipid, an additional GlcNAc residue is added in a similar fashion along with five Man residues by five different ManT's. Here, the Man does not need to be charged on dolichol phosphate; rather, it is initially present as GDP-Man. The resulting structure $Man_5GlcNAc_2$-P-P-Dol is then essentially 'flipped' across the ER membrane, such that the carbohydrate portion of the structure is now facing the lumen of the ER rather than the cytoplasm. This reaction is facilitated by a so-called 'flippase' enzyme not yet characterized. The structure is further elongated within the ER lumen by the addition of four more Man residues (initially as Dol-P-Man here) and three Glc residues (initially as Dol-P-Glc), resulting in the completion of the precursor structure $Glc_3Man_9GlcNac_2$-P-P-Dol. It is at this point that the growing glycan chain is transferred from the dolichol

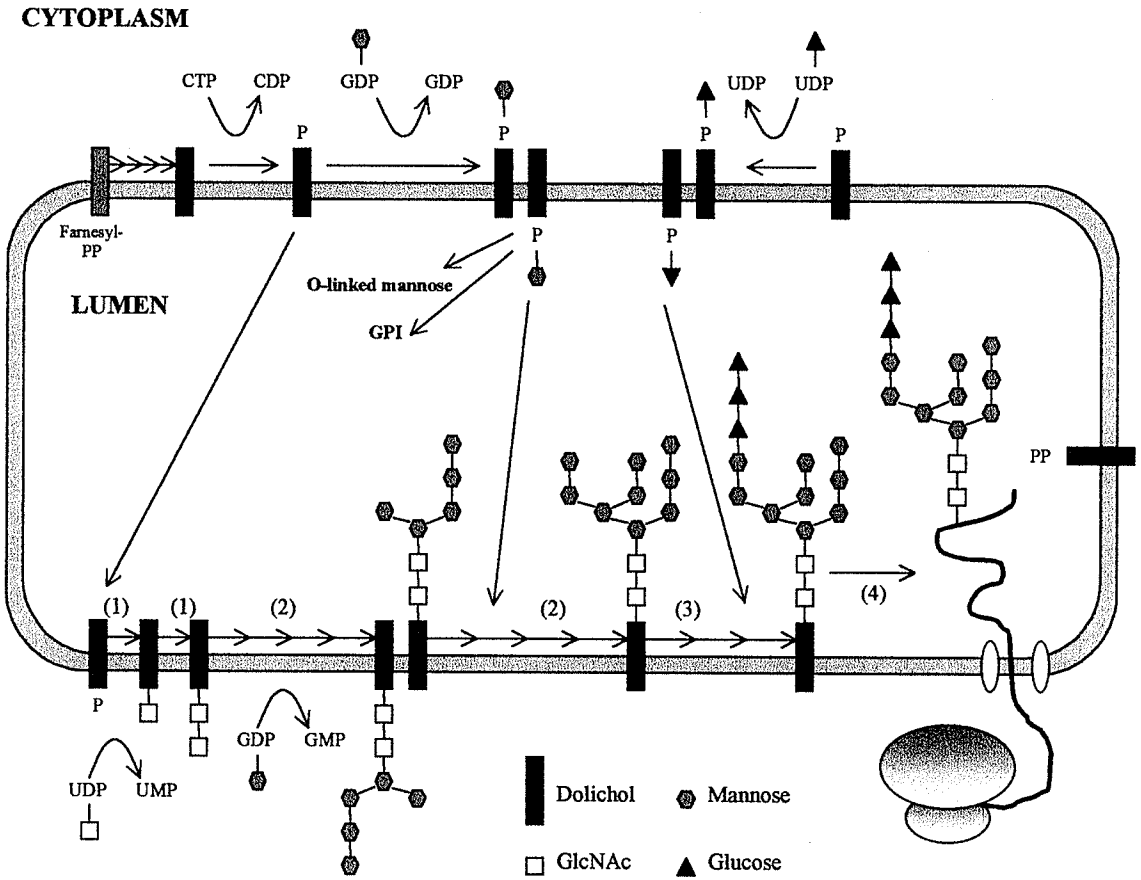

Figure 3. The dolichol phosphate pathway. Shown is the synthesis of the dolichol phosphate sugars in the ER required for the addition of some monosaccharides to the ends of the growing N-linked oligosaccharide core structure. Also depicted is the creation of the core structure itself and its transfer onto a nascent polypeptide chain. Each step in the core synthesis is catalyzed by a different group of GT's, with (1) GlcNAcT's, (2) ManT's, (3) GlcT's, and (4) OST (diagram adapted from Burda and Aebi, 1999).

phosphate molecule onto the Asn residue of a tripeptide referred to as a 'sequon' by an enzyme known as OST, a complex enzyme consisting of eight subunits embedded in the ER membrane. Normally, a sequon consists of the amino acids Asn-X-Ser/Thr in an α-helix at least five amino acids in length, with X being any amino acid except for the helix breaker proline (Gavel and Von Heigne, 1990). Only approximately one third of the protein sequons actually become glycosylated, possibly due to the inaccessibility of the OST to the Asn residue of some sequons as a result of protein folding. Precursor N-linked carbohydrate structures that do not become attached to a protein are cleaved off the dolichol phosphate and are transported to lysosomes, where they are degraded (Cacan *et al.*, 1992).

If attachment to a protein sequon does occur, the carbohydrate structure is then deglycosylated to some degree. Firstly, the membrane-bound enzyme glucosidase I hydrolyzes the external glucose residue of the carbohydrate structure, followed by the excision of the remaining two glucose residues by the soluble heterodimeric glucosidase II. Up to two Man residues are then cleaved by two ER-specific α-mannosidases. If this all occurs in a correct fashion, the glycoprotein is now transferred from the ER to the Golgi apparatus for further glycosylation and differentiation. These deglycosylations may seem to be counterproductive considering that the intent is to synthesize a glycan here rather than break it down, but they actually constitute a key step in the determination of the correct folding of the protein to which the carbohydrate is attached. In effect, the cleavage of the glucose residues is a key step in controlling the quality of the protein before it leaves the ER (Parodi, 2000).

Essentially, when proteins enter the ER, they are modified in several ways besides N-glycosylation, for instance, by having their signal peptides cleaved, obtaining secondary structure characteristics such as disulfide bonds, and eventually attaining their proper tertiary and sometimes even quaternary structures (Parody, 2000). If any of these covalent modifications does not occur as required, a non-functional protein could be the result. As a consequence, the cell has come up with an ingenious way of utilizing glycosylation status to distinguish these malformed proteins from the correctly folded ones, a process commonly known as the 'quality control' of protein folding (see Figure 4) (Hurtley and Helenius, 1989). Following the deglycosylation steps, if the glycoprotein is not properly folded, the Manα1,2Manα1,2Manα1,3 arm on the carbohydrate portion to which the three glucose residues were previously attached is recognized by a specific glucosyltransferase (UDP-Glc:glycoprotein glycosyltransferase; GluT) that adds on a single glucose residue. It has been determined that GluT recognizes only denatured proteins, as conformationally-intact proteins are not affected by this enzyme (Trombetta *et al.*, 1989) and that the denaturation of the protein exposes protein domains required for GluT recognition rather than rendering the oligosaccharide accessible to GluT (Sousa *et al.*, 1992). The actual mechanism by which GluT is able to recognize only denatured proteins is still unclear (Parody, 1998). The single glucose residue added onto the structure by GluT then allows the glycoprotein to be recognized by one of two homologous chaperones in the ER, calnexin or calreticulin. Both of these lectin-like chaperones act in a similar fashion by binding to the misfolded glycoproteins and preventing their release from the ER. Glucosidase II regulates this binding by hydrolyzing the Glc moiety added on by the GluT, with the GluT adding on an additional

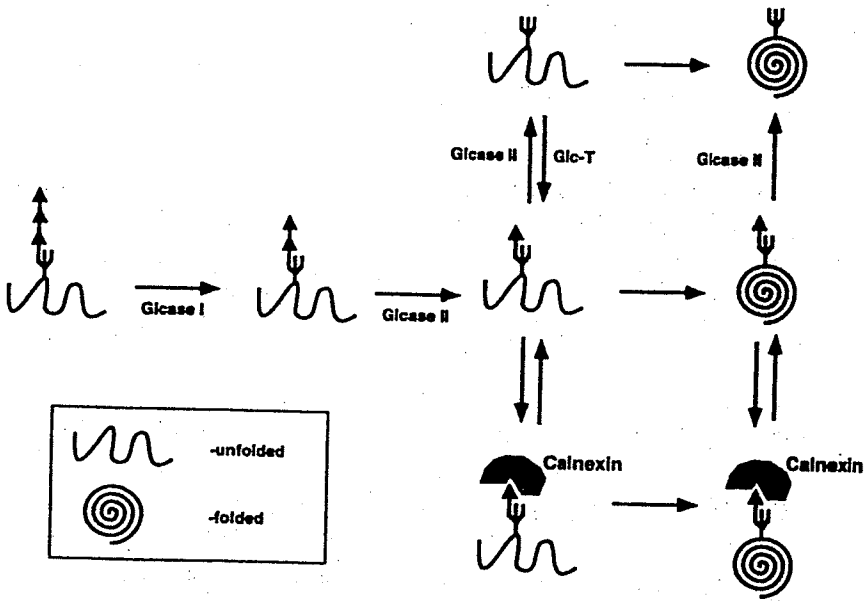

Figure 4. The function of calnexin in glycoprotein quality control. Following the removal of Glc residues (▲) by glucosidases (Glcase) I and II, if the glycoprotein is in a misfolded state, it is reglucosylated by a glucosyltransferase (Glc-T). Calnexin binds to this Glc residue and retains the glycoprotein in the ER for folding (diagram taken from Varki *et al.*, 1999).

Glc if the protein is still not properly folded. Release of the glycoprotein from the ER occurs when the protein has been deemed to be properly folded by the lack of further recognition of the formerly misfolded protein by GluT and subsequent release from calnexin or calreticulin. The glycoprotein is then able to be translocated across the ER membrane, after which it is transported to the Golgi apparatus and the carbohydrate-specific GT reactions can occur.

B. Glycan Differentiation: Modification of the Precursor Structure by the Golgi-Bound Glycosyltransferases

Following the creation of a precursor structure in N-linked glycoproteins and the addition of GalNAc to serine or threonine residues in O-linked glycoproteins, the glycans are able to undergo specific carbohydrate additions in the Golgi apparatus, as mediated by the GT enzymes. Initially, the properly folded N-linked glycoprotein is transported from the ER to the Golgi, an energetically unfavorable task considering that the highly hydrophilic carbohydrate must be transported across the hydrophobic lipid membrane of the Golgi. The actual mechanism by which this occurs is still unknown (Krag, 1998), but it has been suggested that specific proteins may be involved in the process (Rush and Waechter, 1995). The glycoprotein precursor, now within the *cis* compartment of the Golgi, is then able to undergo specific GT-mediated reactions as it moves through the various Golgi cisternae that can result in a tremendous variety of final carbohydrate structures (refer to Figures 5 and 6). For instance, if the highly mannosylated structure present in the precursor is inaccessible to the Golgi GT's or one or more mannose residues becomes phosphorylated, further processing of the carbohydrate structure is

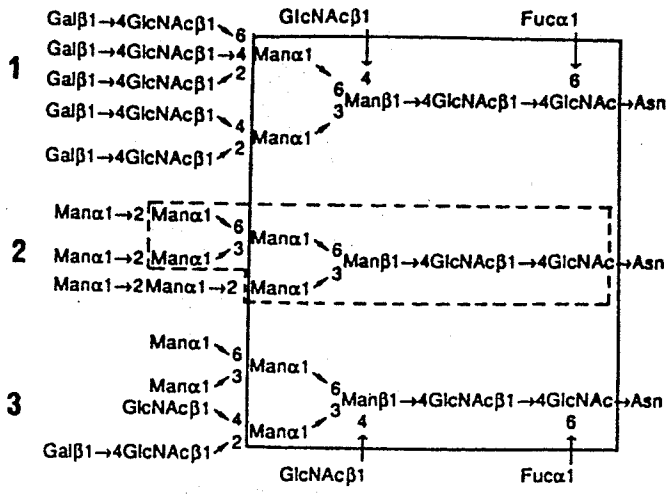

Figure 5. The subgroups of N-linked glycan chains. (1) Complex-type sugar chains; (2) high-mannose-type sugar chains; (3) hybrid-type sugar chains. Shown within the solid line is the pentasaccharide core common to all N-linked glycans. Within the dotted line is the common heptasaccharide of high-mannose-type sugar chains. Structures outside the dotted line can vary in their sugar content (diagram taken from Kobata, 1992).

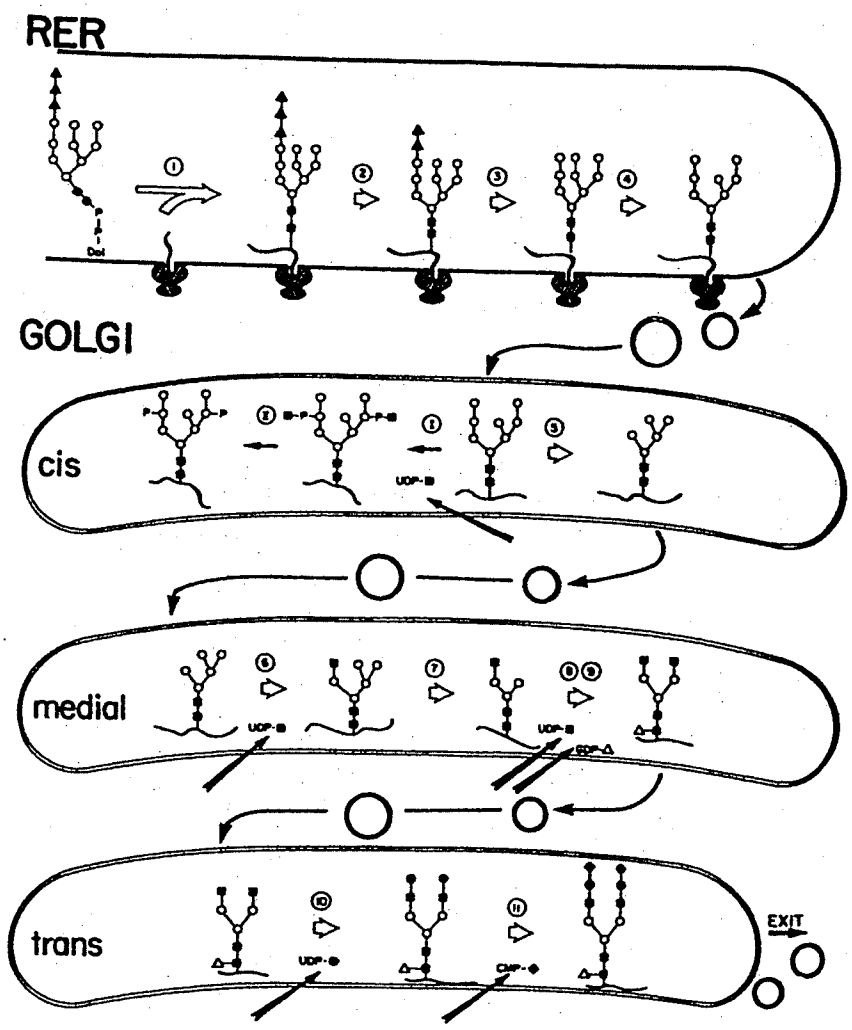

Figure 6. Schematic pathway of oligosaccharide processing on newly synthesized glycoproteins. The reactions are catalyzed by the following enzymes: (1) OST; (2) α-glucosidase I; (3) α-glucosidase II; (4) ER α1,2-mannosidase; (I) GlcNAc phosphotransferase; (II) GlcNAc-1-phosphodiester α-N-acetylglucosamidase; (5) Golgi α-mannosidase I; (6) GlcNAcT-I; (7) Golgi α-mannosidase II; (8) GlcNAcT-II; (9) FucT; (10) GalT; (11) ST. The monosaccharides are represented as follows: GlcNAc (■); Man (○); Glc (▲); Fuc (△); Gal (●); NeuAc (◆). Specific GT's and monosaccharide additions may occur in different Golgi cisternae from those indicated (see later) (diagram taken from Kornfeld and Kornfeld, 1985).

halted and the resulting structure is termed a high-mannose oligosaccharide. On the other hand, if the Golgi α-mannosidase I is able to act upon the precursor, four α1,2-linked Man residues are cleaved from the glycan structure, leaving it open to reaction with GlcNAcT-I, a GT which adds a GlcNAc residue to the free α1,3-linked Man residue attached to the β1,4-Man in the precursor structure. This particular enzyme uses the sugar nucleotide UDP-GlcNAc as the monosaccharide donor. These sugar donors essentially are transported into the Golgi via specific antiporters, allowing for a relatively large pool to be present within the Golgi. Golgi α-mannosidase II is then able to react with the protein-bound hybrid carbohydrate, removing both terminal Man residues (α1,6- and α1,3-linked) from the structure, and further processing and addition of galactose, fucose, and/or sialic acid by specific GT's results in the creation of complex oligosaccharide structures. The reaction of α-mannosidase II is prevented if the glycan structure has already had a bisecting GlcNAc residue added to it by the GT GlcNAcT-III. If reaction of the glycan with GlcNAcT-III does occur, the resulting carbohydrate structure is now termed a hybrid oligosaccharide as it contains aspects of both the high mannose structure and the complex structure and any further branching is prevented. As a consequence, the specific sequence in which the GT's act and bisection occurs (if it occurs at all) can result in thousands of different final glycan structures. Moreover, further modifications of the monosaccharide substrates such as acetylation, sulfation, and phosphorylation can further increase this diversity. Once the glycan structures are complete following further additions by different GT enzymes in the Golgi, the newly synthesized glycoproteins exit the Golgi and are transported to their final destinations.

C. General Reaction Properties, Structure, and Localization of the Glycosyltransferases

Generally, the GT's catalyze the synthesis of glycoconjugates by transferring an activated sugar residue to an acceptor molecule (known as Leloir-type) for either glycan chain initiation or elongation normally in the following reaction, with R being either a free monosaccharide or a monosaccharide linked to another molecule, such as a free glycan chain, a protein, or a lipid:

nucleotide-sugar + R-OH → R-O-sugar + nucleotide

Most GT's require a divalent cation for activity (usually Mg^{+2} or Mn^{+2}) and tend to be the most active in the pH range of 5.0 to 7.0, which reflects the pH values detected within the ER and Golgi apparatus (Varki *et al.*, 1999). Typically, the GT's are usually given trivial names arising from the sugar they attach. For example, the N-acetylglucosaminetransferases (GlcNAcT's) transfer N-acetylglucosamine (GlcNAc), galactosyltransferases (GalT's) transfer galactose (Gal), sialyltransferases (ST's) transfer sialic acid (NeuAc), etc. Furthermore, each GT enzyme of the same type (e.g. ST) is able to transfer a monosaccharide substrate into only one linkage type (known as the 'one enzyme, one linkage' paradigm) (Hagopian and Eylar, 1968; bis Preels *et al.*, 1981) and thus, the action of individual GT's can result in carbohydrates of widely varied structure. In fact, it has been estimated that the synthesis of the carbohydrate portions of glycoproteins and glycolipids can require more than a hundred GT's, with most of these enzymes catalyzing carbohydrate addition to the widely varied terminal regions (Beyer *et al.*, 1981; Sadler, 1984).

Generally speaking, the GT's are type II transmembrane proteins sharing a common basic structure, which consists of a short N-terminal cytoplasmic tail and a 16-20 amino acid membrane-spanning signal anchor peptide that is attached to a lengthy stem region and a C-terminal catalytic domain (see Figure 7) (Paulson *et al.*, 1987). The membrane-spanning domain functions to anchor the enzyme to the membrane of the Golgi apparatus, while the stem region protruding into the Golgi lumen acts as a flexible arm, allowing the attached catalytic domain to come into contact with and further glycosylate carbohydrate structures traversing through the Golgi. Moreover, GT's are actually glycoproteins themselves as it has been demonstrated that they undergo complex-type N-glycosylation (including terminal sialylation) within the Golgi (Costa *et al.*, 1997; Grabenhorst *et al.*, 1998). While most GT's in their active forms have the membrane-bound configuration, soluble GT's have been purified from various body fluids such as milk and serum (Sadler, 1984; Beyer *et al.*, 1981) and increased serum levels of these soluble GT's have been found during disease (Kim *et al.*, 1972) and inflammation (see later) (Lammers and Jamieson, 1989). The soluble forms of GT's may arise from the cleavage of the catalytic subunit from the transmembrane domain by endogenous proteases (Lammers and Jamieson, 1989). It is generally thought that GT's localized outside the Golgi do not perform their transferase functions due to a lack of sugar nucleotide donors (Colley, 1997). Rather, it is believed that they may instead act as carbohydrate-binding lectins in their secreted, soluble forms (Colley, 1997).

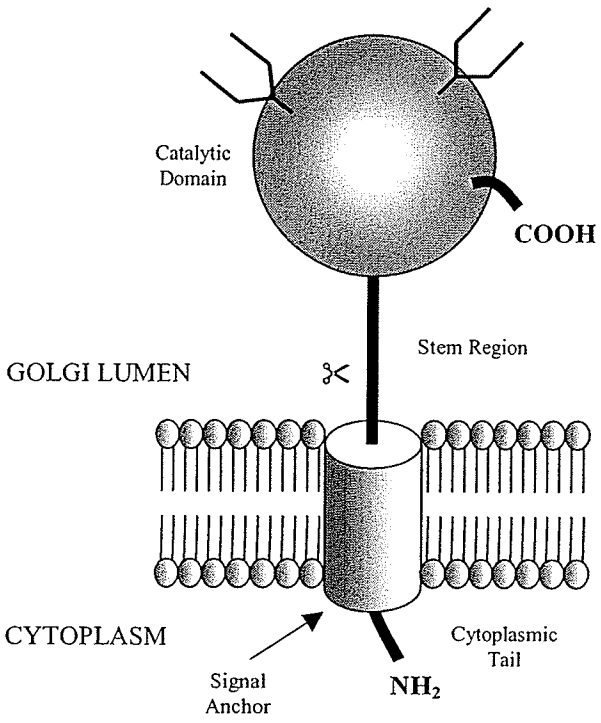

Figure 7. Common GT topology. Deduced amino acid sequences of terminal GT's predict an N-terminal cytoplasmic tail attached to a Golgi membrane-bound signal anchor domain which in turn is bound to an extended stem region and the C-terminal catalytic domain which is oriented within the lumen of the Golgi cisternae. The catalytic domains of GT's may also be glycosylated as shown above, and soluble forms of the enzymes may arise from cleavage at the stem region as indicated (diagram adapted from Paulson and Colley, 1989).

Although they share similar functions, little amino acid sequence similarity has been detected between the catalytic domains of different GT's. Furthermore, no significant homology exists between the GT's and other known proteins (Paulson and Colley, 1989). Despite the general lack of homology, there have been common amino acid sequences detected in homologous binding sites for certain GT's. For instance, the catalytic domains of ST enzymes contain homologous regions termed sialylmotifs that may aid in the recognition of the donor substrate, CMP-NeuAc (see later) (Paulson and Colley, 1989; Datta and Paulson, 1995). Furthermore, it has been suggested that UDP-Gal may bind to the conserved hexapeptides RDKKND in Galα1,3-galactosyltransferase and RDKKNE in GlcNAcβ1,4-galactosyltransferase (Joziasse *et al.*, 1985, 1989; Joziasse, 1992). And more recently, Kapitonov and Yu (1999) identified the presence of three different so-called 'nucleotide recognition domains' (NRD's) in GT's, forming distinct groups in which the different enzymes can be placed based upon the nucleotide sugar they react with and the type of reaction that occurs. Between identical GT enzymes cloned from different species, the amino acid sequence similarity has been found to be fairly high (80% or greater) within the functional domains with the lowest homology occurring in the stem regions (30-40%) (Paulson and Colley, 1989; Schachter, 1994).

The Golgi apparatus itself is arranged into a series of distinct membrane-bound compartments, namely the *cis*-Golgi network or intermediate compartment, the *cis*, *medial*, and *trans* cisternae (Golgi stack), and the *trans*-Golgi network (TGN) (Teasdale *et al.*, 1994). For the most part, the terminal GT's are localized within the Golgi apparatus with the general arrangement not being unlike that of an assembly line, with

the earlier-acting enzymes generally being present closer to the point of entry of the glycoprotein precursors and later-acting enzymes normally being found further within the Golgi, with a few exceptions. In the 1980s and early 1990s, it was believed that the Golgi GT's are arranged within in the Golgi stacks in a very strict fashion, i.e., specific enzymes to specific compartments. This view suggested that the Golgi α-mannosidases I and II are located within the *cis* and *medial* cisternae, the GlcNAcT-I within the *medial* compartment, the GalT within the *trans* Golgi, and the ST within the *trans* Golgi and the TGN (Colley, 1997). However, work done in the mid-1990s involving the immunocytochemical detection of GT's in HeLa cell lines found that the GT's actually overlap in terms of distribution in the Golgi. In these studies, GlcNAc transferase I and α-mannosidase I were both detected in the *medial* and *trans* Golgi cisternae, while the GalT and ST appeared to overlap in the *trans* cisternae and TGN (Nilsson *et al.*, 1993a; Rabouille *et al.*, 1995). Differences in Golgi GT distribution have also been detected between different cell types (Colley, 1997). The main reason speculating as to why the GT's are indeed not present in a specific set of cisternae is that differential distribution allows more room for error, i.e., if a glycoprotein fails to be modified in one cistern, is has an additional chance to become glycosylated as it traverses through the Golgi. In regards to the actual movement of the precursor structures through the Golgi compartments, it has been believed for some time that the precursors travel through the various Golgi compartments from *cis* through to the TGN by vesicular transport (Bergmann and Singer, 1983; Rothman *et al.*, 1984a; Rothman *et al.*, 1984b) but these particular studies were based upon a cell-free assay system, and hence it is unknown whether or not the same process actually occurs within the cell. No other valid

alternative theories as to how the oligosaccharides are able to traverse the Golgi have been proposed recently.

Another feature of Golgi localization of GT's that has eluded researchers for some time now has been the actual mechanism by which the GT's are retained in specific Golgi compartments. Although a few regions of homology have been detected in specific GT's (see above), none of them suggest the presence of a common Golgi retention signal required for the enzymes' retention in the Golgi apparatus (Colley, 1997). As a result, there have been two main theories postulated as to how the enzymes are able to be retained within specific Golgi compartments. The first theory, known as the oligomerization/kin-recognition model (shown in Figure 8) (Machamer, 1991; Nilsson *et al.*, 1993b), suggests that individual GT's, upon their synthesis in the ER, react with another GT of the same type through interactions in their catalytic regions, essentially forming homodimers. These homodimers then move from the ER to the Golgi and are transported through the Golgi along the default pathway until they encounter oligomers in specific cisterns formed by a mixture of GT's, termed 'kin' enzymes. If the homodimer has a specificity for binding to that particular oligomer, it will attach itself to the structure via its membrane-spanning domains and stalk region. As a consequence, the homodimer will be prevented from moving further along in the Golgi as the size of the oligomer formed will be so large that it will be unable to enter vesicles budding off the cisternal rims for protein transport through the Golgi and hence will be retained within the compartment.

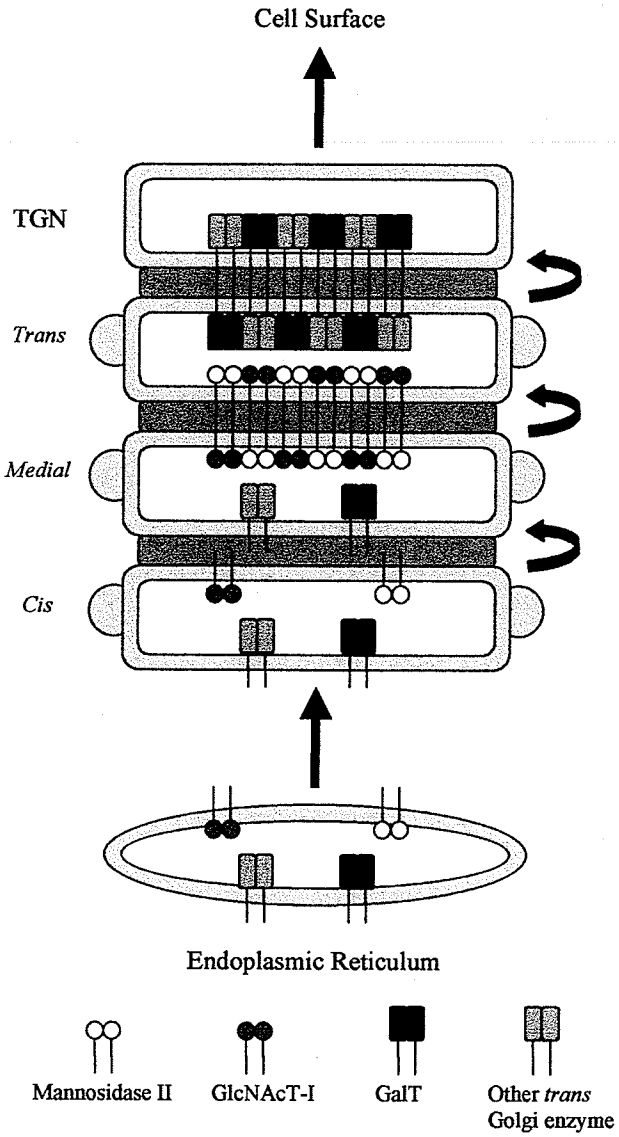

Figure 8. Model for kin recognition. Synthesis and assembly of Golgi enzymes in the ER is followed by their transport along the default pathway until the homodimers interact with the pre-existing kin oligomers via their membrane-spanning domains and stalk regions. The movement of the kin oligomers is halted because their size prevents them from entering the vesicles budding from the cisternal rims, essentially retaining them in specific Golgi compartments (diagram adapted from Nilsson *et al.*, 1994).

The benefit of this theory is that it is able to account for the fact that it is now known that specific GT's are not confined to a particular Golgi cistern. The other main theory, known as the bilayer thickness model (Bretcher and Munro, 1993; Masibay *et al.*, 1993), originated from the observation that the transmembrane domain of GT's can be replaced with a stretch of 17 hydrophobic amino acid residues without affecting the enzymes' localization within the Golgi. However, if 23 hydrophobic amino acids are utilized rather than 17, the GT's instead become retained on the cell surface rather than within the Golgi. These findings were explained by the fact that cholesterol content of the membrane gradually increases moving from the ER through the *cis*, *medial*, and *trans* Golgi compartments, leading to thicker membranes, and that the transmembrane domains of plasma membrane proteins are typically five amino acids longer than those of Golgi-retained proteins. Essentially, this model states that the length of the transmembrane domain determines where in the Golgi a particular GT will become localized. There has been evidence gathered both for and against each of these proposed retention mechanisms (Colley, 1997), but neither alone has been able to account for GT Golgi retention entirely. It is possible that both models may work synchronously, or that there may be another retention mechanism entirely. One recent suggestion of another retention mechanism has been postulated by Grabenhorst and Conradt (1999), who suggest that the cytoplasmic, transmembrane, and stem (CTS) regions of Golgi GT's mediate the sorting and retention of the enzymes into different subcompartmental areas in the Golgi. This idea was based upon the finding that chimeras constructed of human α1,3-fucosyltransferase VI with CTS regions of different early and late acting GT's become retained in different areas of the Golgi. Whether or not this theory (or the two previously described) holds any bearing

to the actual mechanism of Golgi GT retention still remains to be determined.

D. Molecular Cloning of Glycosyltransferase Genes

In recent years, the cloning of GT genes has occurred at a rapidly increasing rate. This has led to the relatively inexpensive synthesis of large quantities of various GT enzymes, allowing it to become feasible to commercially produce them for use in creating specific glycan structures for research. Early on, the most common method used to attempt to clone a specific GT was to first purify the enzyme, after which partial amino acid sequences of the purified protein could be obtained (Kleene and Berger, 1993). By referring to the amino acid sequence, degenerated synthetic oligonucleotides could then be prepared and utilized to screen cDNA or genomic libraries for the specific GT gene. Alternatively, monospecific antibodies could be prepared from the purified GT enzyme and these could be used to screen a library instead. These methods, however, did not readily result in large quantities of GT's being cloned due to the inherent nature of these enzymes. This is because most GT's are present in the cell in minute amounts as the Golgi apparatus is a very efficient functional organelle and thus only a small number of GT molecules are required to glycosylate structures passing through the Golgi, making it extremely difficult to isolate a GT in large enough quantities to purify it (Fukuda *et al.*, 1996). As a result, only relatively abundant GT's such as GalT were cloned early on (Narimatsu *et al.*, 1986; Shaper *et al.*, 1986; Masri *et al.*, 1988) and ST6GalI was the only ST that could initially be cloned due to the low amounts of other ST's in the cell (Weinstein *et al.*, 1987; Wen *et al.*, 1992). The advent of a new technique termed 'expression cloning' by Seed and Aruffo (1987a; 1987b) revolutionized the cloning of

GT's and made it possible to produce recombinant versions of enzymes previously unable to be cloned using older techniques. This method does not require the prior purification of a GT enzymes nor deduction of a partial amino acid sequence. Basically, expression cloning involves the use of specific host cells that do not express the GT of interest yet have the capability to synthesize the acceptor and the activated sugar donor substrates in addition to expressing either the polyoma or SV40 large T antigen, which are necessary for plasmid replication. These host cells are transfected with a cDNA expression library and lectins or antibodies specific to the GT to be cloned are used to detect those transfectants now carrying the GT gene. From host cells expressing the desired antigen, plasmids can then be isolated and divided into pools, after which each plasmid pool can be tested for its capability to express the antigen. Once a particular plasmid pool is found to express the specific GT antigen, it is further divided into smaller subpools which are again tested for expression of the GT antigen. Eventually, further narrowing down of the plasmid pool results in the isolation of a cDNA encoding the GT of interest, which can be introduced in bacterial or yeast cells for production of the enzyme

At present, there have been several types of GT's cloned either through traditional methods or expression cloning, including ST's, FucT's, GalT's, GlcNAcT's, ManT's, and GlcT's, among others (Field and Wainwright, 1995). Cloned versions of the GT's have structures similar to those present in their secreted forms which include the catalytically-active C-terminal domain but lack the membrane anchor and stem regions (Field and Wainwright, 1995). Comparisons of recombinant ST's to native versions of

the enzymes have shown that, in general, the recombinants have a lower affinity for the sugar nucleotide CMP-NeuAc and also exhibit differing affinities towards oligosaccharide and macromolecular acceptors (Williams *et al.*, 1995). It was postulated that the main reason for the different kinetic values between native and recombinant ST's cloned in bacterial cells may be that the only difference in structure between the two is that the recombinant versions lack the carbohydrate chains present in all GT's which may confer various biological properties such as substrate affinity upon the enzyme (Williams *et al.*, 1995). This highlights the main benefit of introducing the GT cDNA into yeast or other eukaryotic cells in that these cell types are capable of glycosylating the GT structure, producing an enzyme with a structure closer to the native form. On the other hand, bacterial cells do not contain the glycosylation machinery and hence produce GT's without the bound carbohydrates found in the native enzymes. Nonetheless, although cloned GT's may have kinetic and possibly other properties differing from their native counterparts, the production of recombinant GT's has been an invaluable development for glycobiology as it is now possible to synthesize specific glycans in the laboratory with relative ease for further study.

Chapter II: The Sialyltransferase Subfamily of the Glycosyltransferases

The ST's comprise a subset of the GT family of enzymes that transfer NeuAc residues to the terminal non-reducing positions of glycolipids and glycoproteins. The ST's are the final GT enzymes to act in the addition of residues to a glycan structure and several different ST's are required to synthesize all the known sialylated carbohydrate structures, each catalyzing a different type of NeuAc attachment (Broquet *et al.*, 1991). In eukaryotes, NeuAc is typically present in α2,3- or α2,6- terminal linkages onto Gal residues of N-linked proteins, with Gal-linked α2,4- and GlcNAc-linked α2,6-NeuAc being less common (Broquet *at al.*, 1991; Paulson *et al.*, 1984). In O-linked glycans, the above linkages occur, in addition to α2,6-linked NeuAc on terminal GalNAc residues and the more uncommonly found α2,8- and α2,9-terminally linked NeuAc (Broquet *et al.*, 1991). With regards to their localization within the Golgi apparatus, generally speaking, the ST enzymes are located in the latter Golgi compartments, namely the *trans* Golgi cisternae and the TGN (Carey and Hirschberg, 1981; Roth, 1987). However, it has been determined that this localization is not exclusive as ST's have been detected in such Golgi regions as the *medial* cisternae and some ST's have been found to be oriented lumenally whereas others occur cytoplasmically (Gonotas *et al.*, 1989; Baubichon-Cortay *et al.*, 1986). Structural analyses have shown that the ST's share a similar structural arrangement to the other GT enzymes, having a cleavable stem-bound C-terminal catalytic subunit attached to a membrane by virtue of a hydrophobic membrane-spanning domain. Within the ST subfamily itself there has been virtually no primary amino acid sequence homology detected between the individual ST enzymes except in two distinct regions in the lumenal catalytic domain, termed the L- and S-sialylmotifs, which

constitute a unique feature of the ST gene family (see Figure 9) (Datta *et al.*, 1998). The larger of these two homologous regions, the L-sialylmotif, consists of 48-49 amino acids exhibiting >70% homology between homologous ST groups and 40-60% homology between heterologous ST groups, along with eight invariant amino acids including one invariant cysteine. The shorter S-sialylmotif contains two invariant amino acids (with one being a cysteine) in a stretch of 23 residues. However, little information has been obtained on the ST subfamily of enzymes with regards to secondary protein structure, mechanism of catalysis, and cellular regulation of transcriptional expression (Harduin-Lepers *et al.*, 1995).

One additional feature of ST's that has eluded researchers for quite some time has been the nomenclature to be adopted for this group of enzymes. Until recently, there has been no consensus on a naming system to be used for the ST's, and as a result, multiple names often appeared in the literature in reference to identical enzymes, creating confusion. In 1996, Tsuji and associates described a systematic form of nomenclature to be used for the ST's similar to that used in naming other GT enzymes to help solve this problem (Tsuji *et al.*, 1996). The goal of this system was not to provide a name for each ST completely describing its functional properties but to instead have a unique name for the enzyme produced by each unique ST gene. This method is the currently accepted system of ST nomenclature used in glycobiology. Basically, four distinct elements make up the scheme ST, x, y, and z, wherein ST denotes the ST family, x is the carbon on the

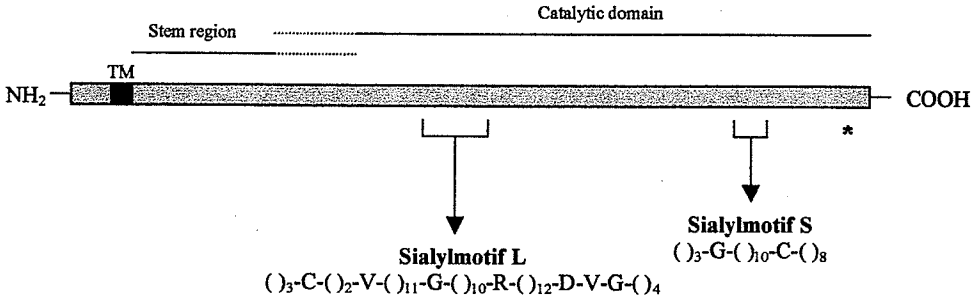

Figure 9. Location of homologous sialylmotif regions within a typical ST enzyme. The L-sialylmotif region consists of 48-49 amino acids and may exhibit > 70% homology among ST family members. The S-sialylmotif is smaller (≈ 23 amino acids) and diverges more among family members. For both, identical residues are indicated and positions of homologous residues are denoted by parentheses. The asterisk indicates the position of the highly conserved sequence H-$X_{(4)}$-E (diagram adapted from Varki *et al.*, 1999).

acceptor sugar to which the NeuAc is transferred (e.g. 6 for NeuAcα2,6Gal), y is the acceptor sugar to which NeuAc is transferred (e.g. Gal, GlcNAc, etc.) and z is a roman numeral given consecutively to each new distinct gene in the subgroup.

The following section will describe properties of three fairly extensively studied ST's, ST3GalII, ST6GalI, and ST3GalIII, as well as a little-known member of the ST subfamily, ST6GlcNAcI, which is the primary focus of this thesis.

A. Examples of Well-Studied Sialyltransferases

i. ST3GalII

The enzyme commonly known as ST3GalII catalyzes the following reaction:

CMP-NeuAc + Galβ1,3GalNAc-R → NeuAcα2,3Galβ1,3GalNAc-R + CMP

with R being an O-linked glycan structure on a glycoprotein, glycolipid, or oligosaccharide possessing a terminal Galβ1,3GalNAc structure, such as the antifreeze glycoprotein, asialofetuin, or the ganglioside G_{M1} (Harduin-Lepers *et al.*, 1995). It has also been determined that ST3GalII has the capability to transfer NeuAc onto the β1,3 hydroxyl group of a Gal residue of Galβ1,3GalNAc α-linked to various aromatic organic molecules such as phenol (Klohs *et al.*, 1981) but that the Galβ1,3GalNAc β-linked to benzene is a poor acceptor for ST3GalII (Klohs *et al.*, 1993). Other studies have shown that lactose (Galβ1,4Glc) and glycans containing terminal Galβ1,3GlcNAc and Galβ1,4GlcNAc also do not react readily with this ST (Harduin-Lepers *et al.*, 1995).

Furthermore, lactosylceramide (Galβ1,4Glcβ1-O-Cer) is a poor acceptor for ST3GalII, as well. The addition of terminal α2,3-linked NeuAc to lactosylceramide requires a different ST, SAT-I, which is an enzyme involved in ganglioside biosynthesis.

ST3GalII activity has been detected in a variety of tissues, including porcine submaxillary gland, human placenta, porcine liver, and rat brain, to name a few, and versions of this ST have been purified from some of these sources (Harduin-Lepers *et al.*, 1995). K_m values determined for ST3GalII purified from some of these organs are vastly different from one source to another, with the ST3GalII from human placenta exhibiting a 20-fold lower affinity for the donor substrate CMP-NeuAc than the same enzyme purified from porcine submaxillary gland and with both enzyme versions showing altered specific activities towards antifreeze glycoprotein (Joziasse *et al.*, 1985). This suggests that there may be several ST's with the capability to transfer NeuAc in an α2,3-linkage onto the O-linked Galβ1,3GalNAc-R structure, and in fact, differing cDNA libraries from different mammals have confirmed that this is likely (Harduin-Lepers *et al.*, 1995). In addition, recombinant forms of ST3GalII have also been prepared and are commercially available. For this particular recombinant, it has been determined that there is only a small difference in K_m compared to native ST3GalII when using antifreeze glycoprotein as acceptor, indicating that the catalytic properties of this enzyme are likely retained fairly well in the recombinant form (Williams *et al.*, 1995).

ii. ST6GalI

The ST enzyme known as ST6GalI catalyzes the following reaction:

CMP-NeuAc + Galβ1,4GlcNAc-R → NeuAcα2,6Galβ1,4GlcNAc-R + CMP

with R representing a free disaccharide or a non-reducing terminal N-acetyllactosamine unit on an N- or O-linked glycan. ST6GalI exhibits high specificity for Type 2 oligosaccharide chains (Galβ1,4GlcNAc) but shows little activity towards Type 1 oligosaccharide chains (Galβ1,3GlcNAc) or the T-antigen (Galβ1,3GalNAc) (Weinstein *et al.*, 1982a). This particular ST has also been shown to transfer NeuAc onto several different disaccharide structures, including lactosamine (Galβ1,4GlcNAc), GalNAcβ1,4GlcNAc, and Manβ1,4GlcNAc (Harduin-Lepers *et al.*, 1995). It has been determined that the main requirements for ST6GalI reaction are the 6-hydroxyl group in β-Gal and the 2-acetamido group of the GlcNAc residue, but that other types of hydroxyl groups can react with this enzyme, as well (Wlasichuk *et al.*, 1993). The Type 2 terminal carbohydrate sequences that are the usual substrate for ST6GalI typically occur in complex N-glycan chains, and studies have determined that optimal reaction occurs with the Gal residue of Galβ1,4GlcNAcβ1,2Manα1,3 branches, with Manα1,6 branches showing considerably less reaction and actually inhibiting NeuAc transfer to Manα1,3 branches if present in large numbers (Joziasse *et al.*, 1985, 1987).

ST6GalI is expressed in a wide variety of tissues and cells, and it has been purified to homogeneity from different animal livers and hepatoma cells where it is

present in the largest quantities as it functions within the liver to sialylate various serum glycoproteins (Harduin-Lepers *et al.*, 1995). A soluble form of ST6GalI lacking the membrane-bound portion has also been purified from bovine colostrum (Paulson *et al.*, 1977; Hesford *et al.*, 1984). In addition, ST6GalI was the first ST enzyme to be cloned, partly facilitated by the large quantities of this enzyme being present in the rat liver from which it was isolated in purified form and used in preparing the recombinant cDNA (Weinstein *et al.*, 1987).

Another soluble form of ST6GalI is present in the serum of mammals whose levels have been found to be modulated by various physiological or pathological changes within the organism it resides, such as cirrhosis, pregnancy, cancer, or induction of the acute phase response (Harduin-Lepers *et al.*, 1995). The acute phase response collectively refers to a set of physiological and biochemical changes that occur following inflammation caused by bacterial infections, exposure to chemical inflammatory agents, or a variety of other pathological changes within an organism (Koj, 1974). Typically, following induction of the acute phase response, there is a 5-10 fold increase in the level of serum glycoproteins such as α_1-AGP, haptoglobin, and fibrinogen (Jamieson *et al.*, 1993), and ST6GalI has been actually found to behave in a similar fashion as increased ST6GalI activity was detected *in vivo* and in rat liver slices following turpentine inflammation (Kaplan *et al.*, 1983). Other liver enzymes have also been found to increase in organisms following inflammation (Jamieson *et al.*, 1987), but only ST6GalI has been observed to be secreted in a catalytically-active form, indicating that it may have a distinct role to play in the acute phase response (Jamieson *et al.*, 1993).

Essentially, prior to inflammation, ST6GalI is bound to the lumenal face of the Golgi membrane, but after the induction of the acute state, the enzyme is cleaved from the membrane by a cathepsin D-like proteolytic activity that releases the ST6GalI into the extracellular space (Lammers and Jamieson, 1988; Jamieson *et al.*, 1993). The actual function of the release of ST6GalI as an acute phase reactant is unknown, but it may function in the sialylation of other acute phase glycoproteins (Jamieson *et al.*, 1993). More recently, it was discovered that levels of ST6GalI are increased in patients suffering from chronic renal failure and may be an indicator of impending delayed graft function post-transplant (Thorne-Tjomsland *et al.*, 2000). Delayed graft function, which occurs in 20-40% of patients with this ailment, is characterized by a requirement for dialysis and a predisposition towards acute transplant rejection (Samaniego *et al.*, 1997). Therefore, ST6GalI could indeed prove to be a major factor used in determining whether delayed graft function will occur following transplant in individuals with this condition (Thorne-Tjomsland *et al.*, 2000).

iii. ST3GalIII

The reaction carried out by ST3GalIII is as follows:

CMP-NeuAc + Galβ1,3(4)GlcNAc-R → NeuAcα2,3Galβ1,3(4)GlcNAc-R + CMP

with R representing an N-linked Type 1 (Galβ1,3GlcNAc) or Type 2 (Galβ1,4GlcNAc) terminal glycan chain. ST3GalIII prefers reaction with the Type 1 chains, and reaction with Type 2 chains occurs with slightly lower efficiency (Weinstein *et al.*, 1982b).

Moreover, it has been determined that rat liver ST3GalIII requires the 3-, 4-, and 6-hydroxyls of the terminal β-Gal for reaction, with the subterminal sugar influencing reaction kinetics, as well. This may account for this ST's ability to react with both Type 1 and Type 2 glycans (Wlasichuk *et al.*, 1993). Furthermore, ST3GalIII does not readily add α2,3-linked NeuAc to the sequence Galβ1,3[NeuAcα2,6]GlcNAcβ (Chandrasekaran *et al.*, 1995). This signifies that the biosynthesis of the disialylated tetrasaccharide NeuAcα2,3Galβ1,3[NeuAcα2,6]GlcNAc-R first involves ST3GalIII-mediated addition of α2,3-NeuAc followed by addition of the side-arm α2,6-NeuAc by ST6GlcNAcI (see later) (Harduin-Lepers *et al.*, 1995). ST3GalIII is also involved in the synthesis of the Lewis blood group determinants sialyl-Le^x and sialyl-Le^a (Harduin-Lepers *et al.*, 1995).

ST3GalIII has been purified to homogeneity from rat liver (Weinstein *et al.*, 1982) and from human colorectal carcinoma cells (Liepkans *et al.*, 1988). In the early 1990's, recombinant forms of ST3GalIII were produced from rat liver (Wen *et al.*, 1992) and human placenta (Kitagawa and Paulson, 1993) cDNA libraries. The cloned rat liver enzyme has been shown to exhibit properties differing somewhat from purified forms (Williams *et al.*, 1995). For example, the native enzyme was observed to have a K_m value half that of the value obtained by the recombinant version when using the oligosaccharide acceptor LNT (Galβ1,3GlcNAcβ1,3Galβ1,4Glc). In addition, the two versions of ST3GalIII appeared to have differing affinities for the macromolecular acceptor human asialo α_1-AGP, and the recombinant also showed a lower affinity for the CMP-NeuAc monosaccharide substrate. Still, these differences did not suggest any

deficiencies in the way the recombinant version of ST3GalIII can be used in the glycosylation of glycoproteins (Williams *et al.*, 1995).

B. ST6GlcNAcI: The Enigma of the Sialyltransferase Subfamily

i. Previous Work on ST6GlcNAcI

The little-studied ST ST6GlcNAcI catalyzes the following reaction:

CMP-NeuAc + NeuAcα2,3Galβ1,3GlcNAc-R →

NeuAcα2,3Galβ1,3[NeuAcα2,6]GlcNAc-R + CMP

where R is a complex-type N-glycan. At present, there have been two major studies performed on ST6GlcNAcI (Paulson *et al.*, 1984; de Heij *et al.*, 1986) and minor references to this enzyme exist in two other publications (Richardson *et al.*, 1994; Harduin-Lepers *et al.*, 1995). The NeuAcα2,3Galβ1,3[NeuAcα2,6]GlcNAc-R structure resulting from side-arm α2,6-linked NeuAc addition by ST6GlcNAcI has been confirmed in bovine prothrombin and fetuin as well as in rat α_1-AGP (Mizuochi *et al.*, 1979; Berman, 1986; Yoshima *et al.*, 1981), but excluding the present study, no efforts have been made to study the kinetics of ST6GlcNAcI using macromolecular acceptors. Furthermore, all attempts to purify this enzyme from rat liver Golgi preparations have failed due to this ST's labile nature. As a result, background information on ST6GlcNAcI is limited and very little is known on its preferred substrates, mechanism and regulation of catalysis, or the physiological and biochemical functions of the structure produced by this enzyme. In addition, there is nothing in the literature that

suggests a function for the oligosaccharide produced by this enzyme. Since sialylated molecules have been shown to have important physiological functions, a study of this structure and the ST that produces it is of great importance.

The study on ST6GlcNAcI performed by Paulson and associates in the mid-1980s involved the use of different human milk oligosaccharides as acceptors, namely LNT, LST_a, LST_b, and DSL, with purified rat liver Golgi fractions presumed to contain ST6GlcNAcI to examine reaction characteristics (structures shown in Figure 10) (Paulson *et al.*, 1984). Firstly, the researchers attempted to find the specific carbohydrate substrate for ST6GlcNAcI. To do so, ^{14}C-labelled products obtained by reaction of LST_a and LST_b with Golgi ST's were identified by descending paper chromatography, and both products were found to cochromatograph with authentic DSL (Paulson *et al.*, 1984). Following treatment of the two products with Newcastle disease virus sialidase, which cleaves the NeuAcα2,3Gal linkage but not the NeuAcα2,6GlcNAc linkage (Drzeniek, 1973), the ^{14}C-labelled DSL obtained using LST_a as substrate was converted to a product with the same migration pattern as LST_b (Paulson *et al.*, 1984). HPLC and methylation analysis of the sialidase-treated product confirmed the presence of the NeuAcα2,6GlcNAc linkage. This suggested that LST_a served as a specific substrate for a Golgi ST that created a NeuAcα2,6GlcNAc linkage. No radioactively labeled species were observed after sialidase digestion of the product formed from reaction of LST_b with the Golgi ST's.

LNT Galβ1,3GlcNAcβ1,3-L

LST_a NeuAcα2,3Galβ1,3GlcNAcβ1,3-L

LST_b
Galβ1,3GlcNAcβ1,3-L
| α2,6
NeuAc

DSL
NeuAcα2,3Galβ1,3GlcNAcβ1,3-L
| α2,6
NeuAc

Figure 10. Structures of human milk oligosaccharides. Terminal structures of LNT, LST_a, LST_b, and DSL are shown. For each oligosaccharide, the remainder of the structure is Galβ1,4Glc (lactose, L) (Paulson *et al.*, 1984).

Following this, LST_a was utilized as a specific acceptor substrate to analyze the reaction properties of the ST6GlcNAcI (Paulson *et al.*, 1984). It was observed that this enzyme displayed a broad pH optimum with little change in activity from pH 5-7. Optimal activity was found to occur with 0.15 M NaCl or 20 mM $MnCl_2$, and the presence of both salts did not result in additive enzyme catalysis. The K_m value determined for CMP-NeuAc was 0.3 mM and the K_m for LST_a was 0.11 mM. Inhibition of the ST6GlcNAcI enzyme occurred more readily with CTP than CDP.

The researchers also set out to determine the order of reaction of the two ST's (ST3GalIII and ST6GlcNAcI) that create the carbohydrate structure NeuAcα2,3Galβ1,3[NeuAcα2,6]GlcNAc-R. When LNT was used as a substrate for the purified Golgi, analysis of the products revealed that LST_a and DSL were present, but that LST_b was not, indicating that the NeuAcα2,3Gal sequence was likely being synthesized before the NeuAcα2,6GlcNAc sequence. All in all, these and other results reported in this publication demonstrated that (1) ST6GlcNAc requires the glycan structure NeuAcα2,3Galβ1,3GlcNAc (as found in LST_a) to add α2,6-linked NeuAc to the GlcNAc residue and (2) the addition of α2,3-linked NeuAc by ST3GalIII to the terminal position of Galβ1,3GlcNAc (as in the LNT structure) must take place before ST6GlcNAcI reaction can occur (Paulson *et al.*, 1984).

The other major study on ST6GlcNAcI, performed by de Heij and associates two years later (de Heij *et al.*, 1986), produced results similar to those obtained in the Paulson study. Once more using human milk oligosaccharides as ST substrates, HPLC and

methylation analysis of the sialylated products again demonstrated that ST3GalIII and ST6GlcNAcI act in a highly preferred order in which the ST3GalIII adds α2,3-linked NeuAc to the carbohydrate sequence Galβ1,3GlcNAc before ST6GlcNAcI reaction (de Heij *et al.*, 1986). Moreover, the ST6GlcNAcI was observed to have a marked preference for the terminal structure found in LST_a (α2,3NeuAcGalβ1,3GlcNAc-R) as opposed to those in LNT (Galβ1,3GlcNAc-R) or the structure GlcNAc-R. The one important new finding determined in this study was that the same or very similar versions of ST6GlcNAcI exist in regenerating rat, normal rat, fetal calf, rabbit, and human liver and human placenta as homogenates of all these tissues were used assayed for ST6GlcNAcI activity, indicating that ST6GlcNAcI may be ubiquitous in mammalian species (de Heij *et al.*, 1986). This is in contrast to the Paulson study in which only normal rat liver ST6GlcNAcI was studied.

Although the Paulson and de Heij studies uncovered important information on the kinetics and properties of ST6GlcNAcI with respect to oligosaccharide acceptors, to properly replicate *in vivo* conditions, reaction kinetics for ST enzymes must be determined using macromolecular acceptors. The following chapter outlines the methods used in the current study to prepare macromolecular acceptors for ST6GlcNAcI that were utilized to study reaction kinetics of the enzyme.

Chapter III: Introduction to Current Work on ST6GlcNAcI

The primary aim of this thesis has been essentially to examine the reaction kinetics of ST6GlcNAcI utilizing macromolecular acceptors prepared from bovine glycoproteins as substrates. As no purified or cloned version of ST6GlcNAcI is available, a suspension of rat liver Golgi presumed to contain this enzyme was prepared using the methods of Leelavathi *et al.* (1970) as modified by Moreman and Touster (1986) and this was used as the source of ST6GlcNAcI. However, the lack of a naturally occurring glycoprotein substrate feasible for lab use with a specificity for ST6GlcNAcI presented an even greater challenge. Basically, ST's and other GT enzymes can be assayed *in vitro* using one of two types of carbohydrate acceptor substrates, oligosaccharide or macromolecular. Oligosaccharide acceptors, as the name suggests, are purely carbohydrate in nature (such as the human milk oligosaccharides LNT and LST_a) and are useful for determining the linkage types made by a particular GT enzyme when used as substrates. However, free oligosaccharide structures very rarely if at all act as substrates for GT enzymes *in vivo*, and hence reaction kinetics determined *in vitro* using oligosaccharide acceptors may be vastly different from those occurring in natural systems. Macromolecular acceptors, conversely, are composed of proteins containing attached carbohydrate moieties and can be prepared in the lab by modifying existing glycoproteins (e.g. through desialylation, etc.). These acceptors are the best acceptors for GT enzymes as they most closely approximate the true substrates *in vivo* of the GT under study and thus the most accurate kinetic values can be determined when using these substrates. Macromolecular acceptors are not very good for use in determining sugar linkage types, however. As noted in the previous chapter, bovine prothrombin and fetuin

and rat α_1-AGP have been found to contain the terminal sequence NeuAcα2,3Galβ1,3[NeuAcα2,6]GlcNAc-R, making any of them a candidate for use as a macromolecular acceptor for ST6GlcNAcI. Although they contain the proper glycan structure, bovine prothrombin and rat α_1-AGP were disregarded immediately as the quantities of these glycoproteins required for kinetic studies would have been economically unfeasible. On the other hand, bovine fetuin was found to be relatively inexpensive for the large quantities required, but its glycan structure requires several additional processing steps to refashion it into a macromolecular acceptor specific for ST6GlcNAcI, unlike the simple desialylations that would have be performed on the prothrombin or rat α_1-AGP. The following section outlines characteristics of the two glycoproteins chosen for modification into macromolecular acceptors, fetuin and another bovine glycoprotein, α_1-AGP, as well as outlining the steps used in refashioning the glycoproteins into ST6GlcNAcI-specific acceptors.

A. The Bovine Glycoproteins used in Acceptor Preparation

i. Fetuin

Fetuin, the most abundant glycoprotein in fetal calf serum, was one of the first glycoproteins to be isolated and characterized (Edge and Spiro, 1987; Green *et al.*, 1988). It has been demonstrated that the bovine fetuin is structurally and biologically related to the human plasma glycoprotein α_2-HS, and fetuin has long been used as a model for the study of glycoprotein structure and biosynthesis (Green *et al.*, 1988). Bovine fetuin consists of a single highly glycosylated polypeptide chain and has a molecular weight of 48 kDa. Moreover, there are six carbohydrate moieties/fetuin molecule, with three being

O-glycosidically linked to serine or threonine and three N-glycosidically linked to asparagine (Spiro and Bhoyroo, 1974). These carbohydrate chains make up 24% of the molecular weight of fetuin (Wang *et al.*, 1998). As is the case for most secretory proteins, fetuin is synthesized with an 18 amino acid signal peptide on membrane-bound polyribosomes by the liver while undergoing cotranslational N-glycosylation (Johnson and Heath, 1986). The glycoprotein is O-glycosylated posttranslationally (Johnson and Heath, 1986). The complete fetuin carbohydrate structure is composed of 20% O-linked carbohydrate, which includes various di-, tri-, tetra- and hexasaccharide structures (Green *et al.*, 1988; Edge and Spiro, 1987), but the majority is composed of N-linked glycans (80%) (Green *et al.*, 1988). It has been determined that the majority of Asn-linked oligosaccharides on fetuin are tribranched, containing three peripheral branches (Green *et al.*, 1988). Small fractions of these tribranched N-linked glycans have been found to contain the structure NeuAcα2,3Galβ1,3[NeuAcα2,6]GlcNAc-R (Green *et al.*, 1988), making fetuin a possible candidate as a macromolecular acceptor for ST6GlcNAcI. However, the presence of O-linked carbohydrate chains and β1,4-linked Gal residues in the N-linked glycans (Townsend *et al.*, 1989) prevent the asialo- version of fetuin from being used as a specific ST6GlcNAcI acceptor due to the possible side reactions that can occur when using a mixed enzyme source as rat liver Golgi. Further processing of this glycoprotein is required to prepare a fetuin macromolecular acceptor with a distinct specificity for ST6GlcNAcI.

ii. α_1-AGP

α_1-AGP is an abundant glycoprotein found in the plasma of humans and various animals (Tamura *et al.*, 1989). The human version of this glycoprotein has been determined to be an acute phase protein, increasing 2- to 4-fold in cases of inflammatory disease, pregnancy, and cancer (Iwata *et al.*, 1987; Iwata *et al.*, 1989). Furthermore, the complete amino acid sequence of human α_1-AGP has been determined as well as the carbohydrate structure (Tamura *et al.*, 1989). In contrast, the protein and carbohydrate structures of the 42-kDa bovine version of α_1-AGP have not been characterized, and the glycoprotein as a whole is actually very poorly studied (Hunter and Games, 1995). However, bovine α_1-AGP was selected for use as a potential macromolecular acceptor for ST6GlcNAcI for several reasons; firstly, this particular glycoprotein is relatively inexpensive, making it an ideal choice for use in synthesizing large quantities of ST acceptor in the lab; secondly, it has a very high carbohydrate content, which at 26.6% is actually the highest found in the bovine plasma proteins (Tamura *et al.*, 1989; Hunter and Games, 1995); thirdly, a large portion of bovine α_1-AGP's glycan fraction has been found to be composed of sialic acid (Yoshima *et al.*, 1981), an important fact if it is to be used as an acceptor for a ST; and fourthly, this glycoprotein exhibits a very high solubility in water and other polar organic solvents (Iwata *et al.*, 1989), which is ideal as an aqueous medium is used in each step of ST6GlcNAcI acceptor preparation. Despite the obvious features that make bovine α_1-AGP an attractive candidate as a potential macromolecular acceptor for ST6GlcNAcI, no references in the literature exist with respect to the proportions of N- and O-linked glycans in this molecule, whether or not there are structures that would interfere with quantitation of ST6GlcNAc reaction if left

intact (e.g. N-linked β1,4-linked Gal residues with a potential to react with ST6GalI in the rat Golgi preparation), or even if the required ST6GlcNAcI acceptor structure NeuAcα2,3Galβ1,3GlcNAc-R actually exists in this glycoprotein. However, bovine α_1-AGP was selected as a potential ST6GlcNAcI acceptor in the current study with the intent that the steps utilized in 'screening' the carbohydrate structures at each step in acceptor preparation would elucidate the answers to these questions and determine whether or not bovine α_1-AGP can indeed make a suitable macromolecular acceptor for ST6GlcNAcI.

B. Procedure for Preparing Macromolecular Acceptors for ST6GlcNAcI

Essentially, the procedure adopted to produce an acceptor for ST6GlcNAcI using bovine fetuin and α_1-AGP was based upon chemical and enzymatic 'refashioning' of the existing carbohydrate structures to produce the acceptor structure NeuAcα2,3Galβ1,3GlcNAc-R in the glycoproteins (refer to Figure 11 for a schematic depiction of the procedure). Ideally, when performing a glycan modification of this sort, it is important to analyze the carbohydrate structures produced at each step by an analytical method such as HPAEC-PAD or mass spectrometry before proceeding onto the next step in the alteration. However, this makes for a very time-consuming process although it basically guarantees the correct glycan composition at the end. To quickly produce ST6GlcNAcI acceptors in this study, a novel approach was taken. After each modification step, the acceptors were assayed against one or more recombinant ST's to determine whether removal or addition of a particular sugar residue was complete based on the presence or absence of significant reaction with the ST. For example, following

removal of NeuAc residues by dilute acid hydrolysis (producing the *As*, or asialo-, versions of the acceptors), both potential acceptor glycoproteins were treated to remove O-linked carbohydrate chains using a modified version of Carlson degradation (Likhosherstov *et al.*, 1990). Small aliquots of the fetuin and α_1-AGP treated in this way (denoted as *As-O* in this study, i.e. the asialo- versions of the glycoproteins with O-linked glycans removed) were assayed with recombinant ST3GalII to determine if hydrolysis of the O-linked residues was complete. This was based upon negligible reaction of the *As-O* acceptor forms with the ST as compared to the reaction of the enzyme with the *As* glycoproteins which contained intact asialo- O-glycans. If this was found to occur, O-glycan removal was deemed to be complete and the next step in the carbohydrate modification was carried out. Actually, the importance of the results obtained from ST testing of the α_1-AGP acceptors during synthesis was twofold – firstly, it indicated whether complete removal or addition of a saccharide in the structure had been accomplished (as with fetuin), and secondly, it gave information on the carbohydrate composition of the α_1-AGP, which, as indicated in the previous section, is sparse at best. For instance, the assaying of the *As* and *As-O* α_1-AGP with ST3GalII resulted in consistently low reaction values for both acceptor versions, indicating that O-linked carbohydrates likely are not present in bovine α_1-AGP, and after sufficient testing, allowed the Carlson degradation step to actually be left out for the α_1-AGP. Acceptor preparation on the two glycoproteins was carried out in this fashion until the final step in producing the correct structure NeuAcα2,3Galβ1,3GlcNAc-R was reached and recombinant ST testing indicated that the structure was likely correct. The completed macromolecular acceptors were then assayed for ST6GlcNAcI activity using a rat liver

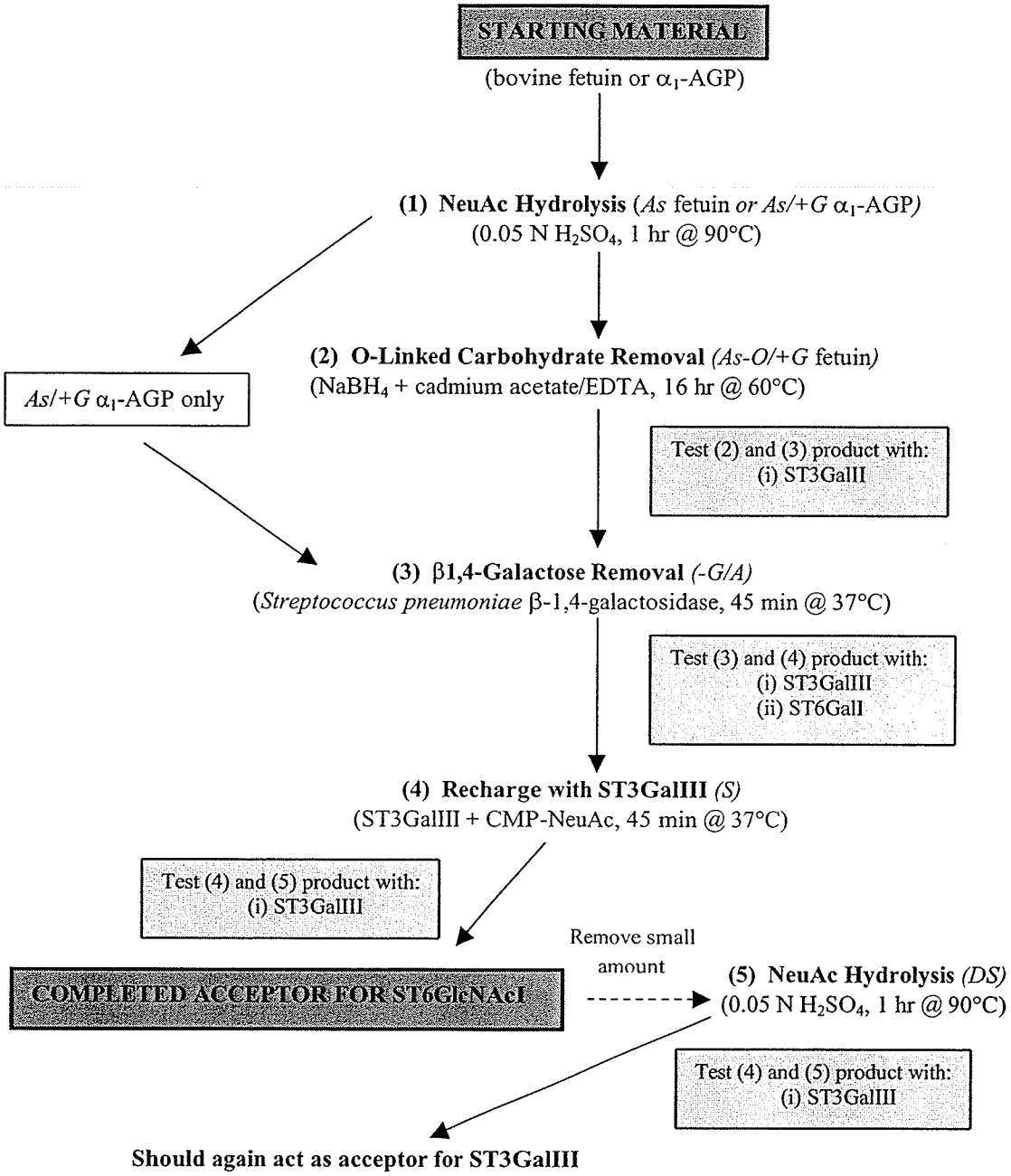

Figure 11. General scheme for the preparation of macromolecular acceptors for ST6GlcNAcI from bovine fetuin and α_1-AGP. The designations given to the forms of the acceptors produced at each step are given in brackets. Samples must be dialyzed and lyophilized after all of steps (1) – (5).

Golgi suspension and K_m and V_{max} values were determined. The one main drawback of the ST testing strategy utilized in this study to confirm the expected acceptor structure is that the correct carbohydrate composition is not guaranteed, i.e. it does not give specific information in regards to the actual linkage types and sugars present as other analytical methods do. It does, however, offer a unique way of determining a 'best estimate' of glycan structure and allows macromolecular acceptor preparation to proceed much more rapidly than with other methods.

The main goal of this thesis has been to provide data on the kinetics of rat liver ST6GlcNAcI reaction with macromolecular acceptors. However, valuable information has also been determined with respect to developing a unique method to be used in the preparation of macromolecular acceptors for GT enzymes as well as providing data on the little-studied carbohydrate makeup of bovine α_1-AGP. Hopefully, the information obtained in this study will shed some light on the workings of a little-known member of the ST subfamily of enzymes.

MATERIALS

Bovine fetuin prepared by the Pederson method was obtained from Life Technologies (Gibco BRL) (Burlington, ON) and bovine α_1-AGP was obtained from Sigma Chemical Company (St. Louis, MO). CMP-NeuAc was obtained from Sigma. CMP-[4,5,6,7,8,9-^{14}C] NeuAc was purchased from New England Nuclear Corporation (Bedford, MA) at 255.9 mCi/mmol and from Amersham Corporation (Oakville, ON) at 280 mCi/mmol. *Streptococcus pneumoniae* β1,4-galactosidase was obtained from either Calbiochem-Novabiochem Corporation (San Diego, CA) or from Sigma (NOTE: one unit of activity is defined as the amount of β1,4-galactosidase that will catalyze the release of 1.0 μmol of *p*-nitrophenol from *p*-nitrophenol-β-*D*-galactoside [Calbiochem] or *o*-nitrophenol from *o*-nitrophenol-β-*D*-galactoside [Sigma] per minute at 37°C). The *Spodoptera frugiperda* recombinant forms of rat ST3GalII, ST3GalIII, and ST6GalI were purchased from Calbiochem, with the ST6GalI also being obtained from Sigma. Dialysis membrane tubing with MWCO's ranging from 10 – 14,000 Da was purchased from Spectrum (Gardena, CA). Ultrafiltration Amicon YM30 membranes were obtained from Millipore Corporation (Bedford, MA). Two and a half cm diameter Whatman #1 filter paper disks were purchased from Fisher Scientific (Edmonton, AB). The following chemicals were obtained as follows: sodium borohydride, crystalline bovine serum albumin, and PTA were from Sigma; sodium cacodylate and cadmium acetate were obtained from J. T. Baker Chemical Company (Phillipsburg, NJ); EDTA-Na_4 was purchased from Aldrich Chemical Company Incorporated (Milwaukee, WI); solid sodium hydroxide was obtained from Mallinckdrodt Specialty Chemicals Company (Paris, KY);

and Bradford protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Hercules, CA). All other chemicals were obtained from Fisher Scientific.

METHODS

1. Preparation of Macromolecular Acceptors

A. Removal of Sialic Acid Residues

Bovine fetuin prepared by the Pederson method and bovine serum α_1-AGP were dissolved separately in 0.05 N sulfuric acid (1 mL H_2SO_4:5 mg glycoprotein). The mixtures were then heated in a 90°C water bath for 1 hour, after which they were chilled on ice and dialyzed exhaustively at 4°C against several changes of deionized water. Lyophilization was then carried out to completely dry the samples. The samples were first concentrated using an Amicon ultrafiltration membrane with a MWCO of 30,000 Da to reduce the solution volume if necessary prior to lyophilization. This procedure produced the asialo-, or, as denoted in these studies, *As* form of fetuin and the *As* or *+G* form of α_1-AGP.

B. Removal of O-Linked Glycans

Bovine *As* fetuin and initially *As* α_1-AGP as well were treated to remove O-linked carbohydrates using a modified version of alkaline-borohydride hydrolysis (Carlson degradation) (Likhosherstov *et al.*, 1990). This procedure involves the addition of a Cd^{+2} salt along with EDTA-Na_4 to the standard reaction mixture to allow for maximum hydrolysis of the O-linked glycans while minimizing hydrolysis of N-linked carbohydrates and the protein backbone (Likhosherstov *et al*, 1990.). A cadmium acetate/EDTA-Na_4 solution was prepared by mixing equal volumes of 0.1 mL EDTA-Na_4 and 23 mg/mL cadmium acetate solutions. The pH of the resulting mixture was then adjusted to 7-7.5 with glacial acetic acid. Each 10 mg of glycoprotein to be treated was

dissolved in 1.4 mL of deionized water. To this was added an additional 0.2 mL of water, 0.29 mL of the cadmium acetate/EDTA-Na_4 solution, 0.11 mL of 0.5 M sodium hydroxide, 152 mg of sodium borohydride, and 0.1 mL of butanol. The flask was then stoppered and the mixture was incubated at 60°C for 16 hours. The solution was then cooled in an ice water bath, the pH was adjusted to 7-7.5 with 4 N acetic acid, and the final volume was brought up to 10 mL with deionized water. The amounts of the various components used in this procedure were scaled up proportionately if more glycoprotein was to be treated. Following this, the samples were dialyzed exhaustively at 4°C against several changes of deionized water and were lyophilized (with the samples being concentrated with Amicon ultrafiltration membranes before freeze-drying if the volume was high). This O-glycan removal procedure was later deemed to be unnecessary for bovine *As* α_1-AGP as no O-linked chains were detected (refer to Results, section B). However, for bovine *As* fetuin, this produced the asialo- form of the acceptor without O-linked chains, which was denoted as *As-O* or *+G* fetuin. Both the *As* and *As-O* forms of fetuin were then assayed against a commercial O-linked α2,3-ST (ST3GalII) to determine if the O-linked glycans were sufficiently diminished in the *As-O* fetuin.

C. Removal of β1,4-Galactose Residues

15 – 26 mg of bovine *As-O* fetuin and 31 – 44 mg of bovine *As* α_1-AGP were dissolved separately in 0.5 – 2.5 mL sodium cacodylate buffer, pH 6.0. To each of these samples was added 100 μL of *Streptococcus pneumoniae* β1,4-galactosidase. This corresponded to an addition of 105 mU of the Calbiochem version of the enzyme or 145 mU of the Sigma enzyme. The samples were then incubated at 37°C for 30 – 45 minutes.

After incubation, the samples were placed on ice to cool, after which they were dialyzed exhaustively against deionized water followed by lyophilization. This step created the asialodegalacto- forms of bovine α1-AGP and fetuin (with the O-glycans removed), and the acceptors at this step were designated the *–G* or *A* forms. Both the *+G* and the *–G* forms of both the α1-AGP and fetuin acceptors were then assayed against commercial N-linked α2,3- and α2,6-ST's (ST3GalIII and ST6GalI, respectively) to determine if the β1,4-Gal residue removal was successful.

D. Addition of α2,3-Sialic Acid to β1,3-Galactose Residues

2 – 26 mg of bovine *–G/A* fetuin and 18 – 37 mg of *–G/A* α_1-AGP were dissolved separately in 0.3 – 1.7 mL sodium cacodylate buffer, pH 6.0. To the fetuin solution was added 50 –150 μL of 10 mg/mL CMP-NeuAc, and to the α_1-AGP solution 75 – 500 μL of the 10 mg/mL CMP-NeuAc was added. The amount of CMP-NeuAc solution included in each reaction was estimated based on the amount of starting *–G/A* glycoprotein. To facilitate the addition of α2,3-linked sialic acid to the carbohydrate structures of the glycoproteins, 50 – 95.2 μL of the *Spodoptera frugiperda* recombinant form of rat ST3GalIII was added to both glycoprotein mixtures. The samples were then incubated at 37°C for 30 minutes to 1 hour. Following this, the mixtures were placed on ice and 0.3 mL of cold 0.15 M sodium chloride/5 mM sodium azide was added to each reaction to halt any further enzyme activity. To ensure complete cessation of any ST3GalIII activity, the samples were then heated in a 90°C water bath for 10 minutes. The mixtures were cooled on ice again, which was proceeded by exhaustive dialysis at 4°C against deionized water and lyophilization. This final step in the acceptor

preparation produced the α2,3-sialyldegalacto- forms of bovine α_1-AGP and fetuin (with the O-linked chains removed), which were referred to as the *S* forms, or completed acceptor, for ST6GlcNacI. The *A* and *S* forms of the acceptors were then assayed against the same commercial recombinant ST3GalIII used in this step in acceptor preparation to ensure that α2,3-sialylation was complete in the *S* forms. For fetuin, α2,3-sialylation appeared to be complete after one of the above treatments, but the α_1-AGP normally required the above procedure to be repeated 1 – 3 times for α2,3-sialylation to be achieved as evidenced by the results in the testing ST3GalIII assays. Once α2,3-sialylation appeared to be complete, 2 – 4 mg of the complete lyophilized *S* α_1-AGP and fetuin acceptors were taken and re-treated to remove sialic acid residues as in step 1A, with the remainder being set aside. After step 1A was repeated on the small portions, these samples were denoted the *DS* forms of the acceptor. After the *DS* samples were dialyzed and lyophilized, they were once again tested along with their corresponding *S* samples with the ST3GalIII enzyme to determine if they could again act as acceptors of α2,3-NeuAc residues. After the results from this test were deemed to be satisfactory, the remaining *S* α_1-AGP and *S* fetuin that had been set aside were dissolved in 1 M sodium cacodylate buffer, pH 6.0, for use in rat liver Golgi ST6GlcNAcI assays, with the fetuin acceptor being brought to a final concentration of 4.6 mg/mL and the α1-AGP being brought up to 10 mg/mL.

2. Preparation of ST6GlcNAcI from Rat Liver

A. Acquiring Rat Livers

Male Long Evans hooded rats were obtained from Charles River Incorporated (Constance, QB) and were sustained on a diet of standard pelleted rat food and water until required. To obtain livers, rats weighing from 500 – 600 g were anesthetized and sacrificed by ethyl ether inhalation. The chest cavity was opened up, and the livers were perfused *in situ* via the hepatic artery with ice-cold 0.15 M NaCl to remove as much blood from the organ as possible. Once this was complete, the livers were excised, rinsed two times with ice-cold 0.15 M NaCl, and were stored on ice until needed.

B. Preparation of Rat Liver Golgi Membranes

Rat liver Golgi were prepared using the methods of Leelavathi *et al.* (1970) as modified by Moreman and Touster (1986). This method ensures that the final product is free of lysosomal material. All sucrose solutions were made up in 0.1 M potassium phosphate buffer, pH 7.4, with 5 mM magnesium chloride. The freshly perfused rat livers obtained in step 2A were minced over ice and homogenized in 0.5 M sucrose (4 mL/1 g liver) using four strokes of a Potter-Elvehjem homogenizer fitted with a rough pestle at 1000 RPM. The resulting suspension was centrifuged at 600 g_{av} (3000 RPM) in a Ti60 rotor to remove cellular debris and other large particles. The supernatant was saved along with the top 1/3 of the pellet and this was re-homogenized at 1000 RPM using 2 strokes of the homogenizer fitted with a smooth pestle. Ten mL of this homogenate was layered over 16 mL 1.3 M sucrose in ultracentrifuge tubes, and these were centrifuged at 75,000 g_{av} (33,000 RPM) in a Ti60 rotor for 60 minutes. The crude

Golgi membrane bands appearing above the 1.3 M layer were aspirated with a Pasteur pipette and all these homogenates were pooled, after which the concentration was adjusted to 1.1 M sucrose. After adjustment, 10 mL of this crude Golgi suspension was layered over 20 mL 1.25 M sucrose in ultracentrifuge tubes and 4 mL 0.5 M sucrose was placed over this. The tubes were then centrifuged in a SW28 rotor at 80,000 g_{av} (24,000 RPM) for 90 minutes. Following this, the Golgi bands appearing between the 0.5 M and 1.1 M layers were aspirated, these fractions were pooled, and the concentration of the pooled mixture was once again adjusted to 1.1 M sucrose. The discontinuous sucrose density gradient centrifugation step was repeated one additional time. The Golgi membrane bands between the 0.5 M and 1.1 M layers were aspirated as before, and this pooled fraction was diluted 1 in 4 with 0.25 M sucrose. The mixture was then stored on ice at 4°C overnight. The next day, the diluted Golgi membrane suspension was centrifuged at 175,000 g_{av} (40,000 RPM) for in a Ti60 rotor for 30 minutes. The supernatant was decanted, and the resulting Golgi pellets were resuspended in a small volume of 0.25 M sucrose by homogenization. The protein content of the suspension was determined using the method of Lowry *et al.* (1951) as modified by Miller (1959), with bovine serum albumin being used as the standard protein. The suspension was then adjusted to 10 mg/mL, and it was used immediately in enzyme assays as a source of ST6GlcNAcI.

3. Measurement of Sialyltransferase Activity

A. General Assay Procedure

Sialyltransferases were assayed using a modified version of the disc assay procedure developed by Baxter and Durham (1979). Twenty-five μL of the standard reaction mixture containing 45 μg bovine serum albumin, 1.2% Triton CF-54, 120 mM sodium cacodylate (pH 6.0), 6.352 nmol CMP-NeuAc, and enough CMP-[4,5,6,7,8,9-^{14}C] NeuAc to supply 20.6 (New England Nuclear version, 0.0805 nmol) or 25.78 (Amersham version, 0.921 nmol) nCi of radioactivity was combined with 30 μL of acceptor preparation and 25 μL of enzyme source over ice, for a total sample volume of 80 μL. The samples were then incubated for 45 minutes at 37°C. After this, two 25 μL aliquots of each sample were spotted on 2.5 cm diameter disks of Whatman #1 filter paper. The disks were then allowed to dry at room temperature overnight or were dried in a 37°C oven for one hour. The dried disks were washed using a similar procedure to that given in Kaplan *et al.* (1983). The first step entailed a washing for 10 minutes in ice-cold 10% TCA/1% PTA (approximately 10 mL/disk), which was followed by two subsequent 10-minute washings in ice-cold 5% TCA/1% PTA. The disks were then washed for 10 minutes in 2:1 (v:v) ethanol:ether, and finally in ether for 8 minutes. The disks were removed to air-dry, after which they were transferred to individual scintillation vials, 10 mL of ACS scintillation cocktail were added, and they were counted in a liquid scintillation counter. The results were standardized by subtracting dpm values obtained for assay vials containing all the assay components except acceptor from those containing acceptor, and these were termed the Δdpm values. Reaction rates were calculated from these Δdpm values (i.e. using the ratio 11.34 dpm/pmol NeuAc

transferred) and incubation time, and all values acquired using assay mix containing the lower radioactivity New England Nuclear CMP-[4,5,6,7,8,9-^{14}C] NeuAc were corrected up to the values obtained using assay mix containing the more radioactive Amersham CMP-NeuAc.

B. Acceptor Testing Assays using Commercial Sialyltransferases

The bovine fetuin and α_1-AGP ST6GlcNAcI acceptors were tested at various points in their synthesis using commercially-available ST's. ST3GalII, ST3GalIII, and ST6GalI enzymes from various sources (see previously) were diluted 1:100 in 0.1 M sodium cacodylate buffer, pH 6.0, plus 18% bovine serum albumin, and this was used as the 25 μL enzyme source. In addition to the enzyme, each complete reactive assay sample contained 15 – 30 μg of fetuin or α_1-AGP acceptors at various stages in their synthesis and 25 μL of the standard reaction mixture, with the final volume being brought up to 80 μL with deionized H_2O. The assay procedure was carried out as in Step 3A.

C. Commercial Sialyltransferase Time Response Assays

To ensure that the 45-minute incubation time utilized for the acceptor testing assays falls within the linear portion of a time response curve, *As*, *As-O/+G*, and *–G/A* fetuin acceptors were assayed with ST3GalII, ST6GalI, and ST3GalIII, respectively, varying the time of incubation from zero to 120 minutes. Each complete reactive assay contained 30 μg of fetuin acceptor, 25 μL of purified ST3GalII, ST3GalIII, or ST6GalI diluted 1:100 in 0.1 M sodium cacodylate buffer, pH 6.0, plus 18% bovine serum albumin, and 25 μL of the standard reaction mixture for a final volume of 80 μL. The

assay procedure was carried out as in Step 3A with the exception that Δcpm values were calculated instead of Δdpm values, and no further calculations were performed on these values to determine assay response. .

C. Rat Liver Golgi ST6GlcNAcI Assays

The completed, fully tested bovine fetuin and α_1-AGP ST6GlcNAcI acceptors were assayed using the rat liver Golgi membrane preparation as the source of ST6GlcNAcI. Each complete assay sample contained 250 μg of Golgi protein (from 10 mg/mL suspension of Golgi in 0.25 M sucrose in potassium phosphate buffer, pH 7.4) and 50 – 300 μg of the α_1-AGP acceptor or 23 – 138 μg of the fetuin acceptor and 25 μL of the standard reaction mixture, with the final volume being brought up to 80 μL with 1 M cacodylate buffer, pH 6.0. The assay procedure was carried out as in Step 3A. Michaelis-Menten kinetic plots were constructed from the resulting enzyme reaction rates and substrate concentrations. A curve fitting algorithm generated by Kaleidagraph™ (version 4.1) was used for determination of K_m and V_{max} values.

RESULTS

A. Assessment of Commercial Sialyltransferase Time Response Activity

To assess for the creation of the proper macromolecular acceptor carbohydrate structure following chemical or enzymatic treatment, the fetuin and α_1-AGP were assayed with one or more of three commercially available ST's (ST3GalII, ST3GalIII, or ST6GalI) for a 45-minute time period (see below for these results). In order for the Δdpm values obtained from these assays to be used to calculate reaction rates, the 45-minute time point must fall within the linear portion of a time versus assay response curve for the reaction rates to be kinetically valid. For these assays, the three above ST's were assayed with reactive forms of the fetuin acceptor for incubation times ranging from zero to 120 minutes. The time response curves generated from these assays are shown in Figures 12 to 14.

B. Assessment of Removal of O-Linked Carbohydrate Structures in Bovine Fetuin and α_1-AGP

To prepare macromolecular glycoprotein acceptors for the sialyltransferase ST6GlcNAcI, bovine fetuin and α_1-AGP were first subjected to dilute acid hydrolysis to remove sialic acid residues, which was followed by a modified version of alkaline-borohydride hydrolysis (Likhosherstov *et al.*, 1990) that results in the specific cleavage of O-linked glycans while leaving N-linked carbohydrates and the protein backbone intact. Initially, O-glycan removal was first performed on fetuin, which is known to contain three O-glycosidically-linked carbohydrate moieties/molecule (Spiro and Bhoyroo, 1974) as well as on α_1-AGP, on which the complete glycan structure has not been published.

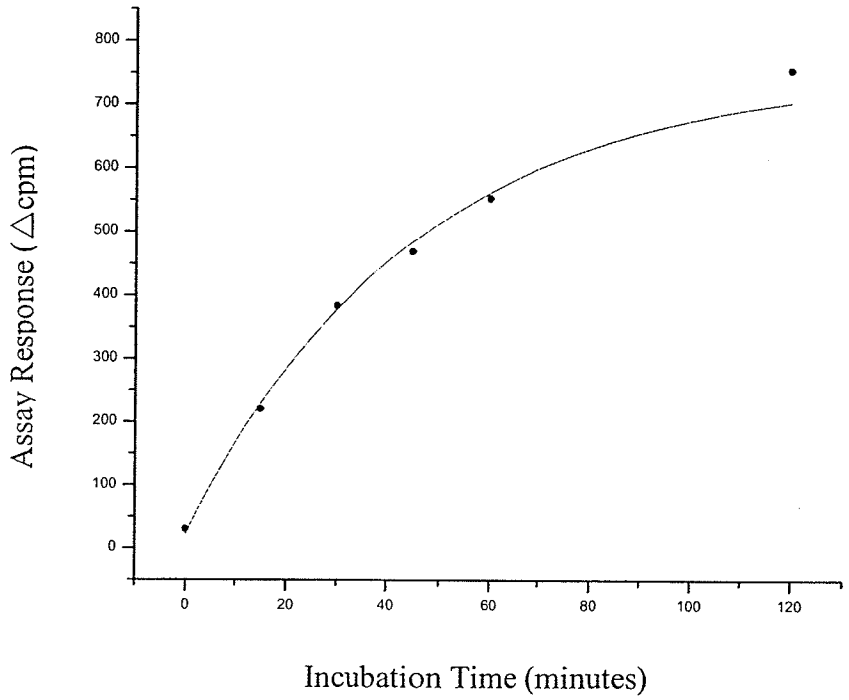

Figure 12. Time response curve of *As* fetuin with commercial ST3GalII. Each data point represents the average of two samples from the same assay.

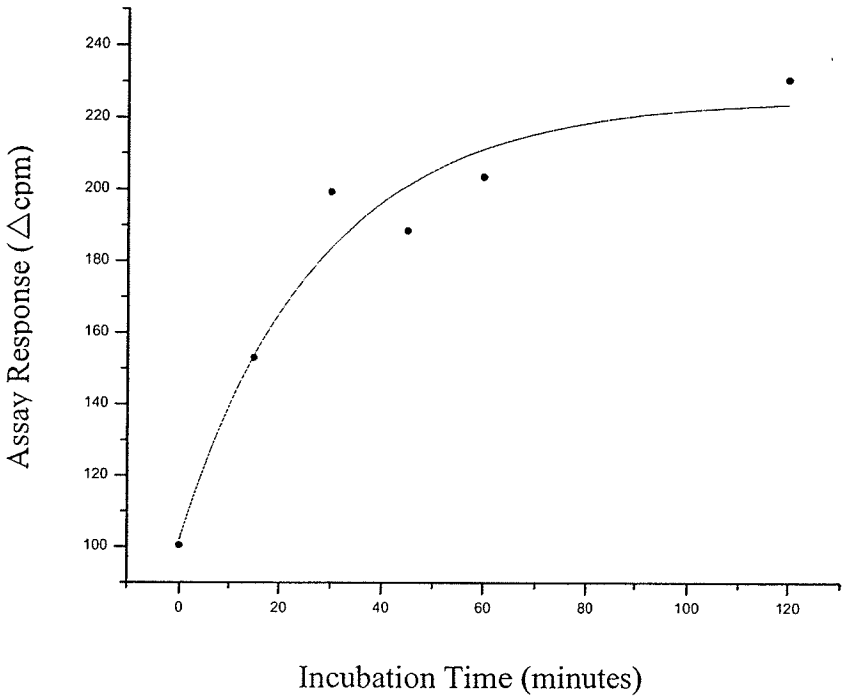

Figure 13. Time response curve of *–G/A* fetuin with commercial ST3GalIII. Each data point represents the average of two samples from the same assay.

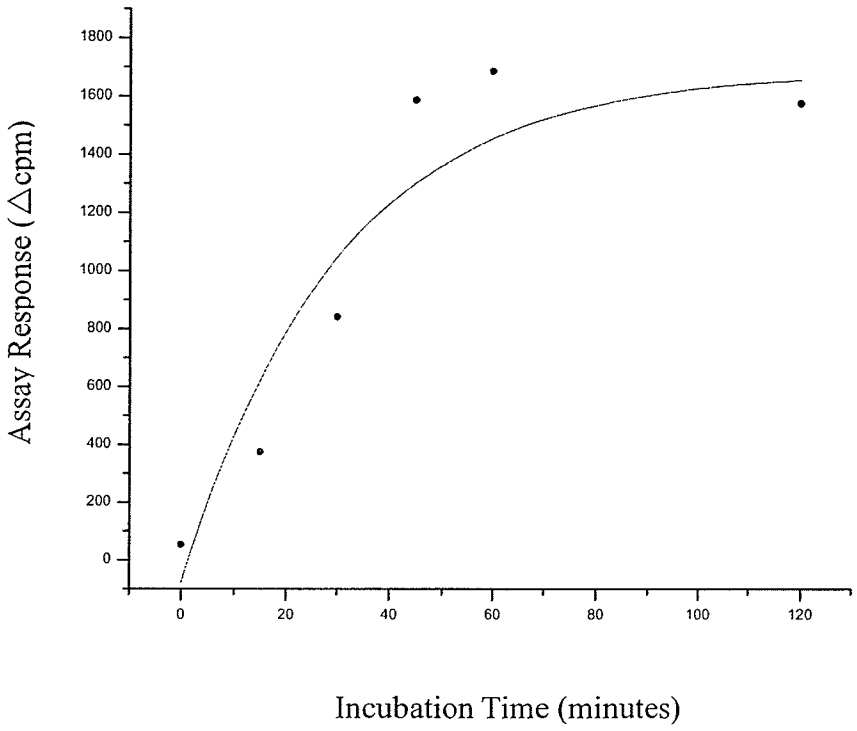

Figure 14. Time response curve of *As-O/+G* fetuin with commercial ST6GalI. Each data point represents the average of two samples from the same assay.

Following these treatments, the *As-O* versions of fetuin and α_1-AGP along with their corresponding *As* glycoproteins were assayed against commercial ST3GalII and reaction rates were calculated as described in Results. ST3GalII has a specificity for the addition of α2,3-linked sialic acid onto the terminal positions of O-linked glycans. Reaction rates obtained in these assays using 15 μg of the *As* glycoproteins should be approximately half of those obtained for 30 μg of the corresponding *As* glycoproteins if O-linked glycans are present (as in fetuin). If O-linked carbohydrate removal has been successful (as expected for *As-O* glycoproteins), reaction rates for both 15 and 30 μg acceptor should be similar in value as well as close to zero and/or consistently low when compared to the values for both amounts for *As* glycoproteins (if O-linked glycans are originally present). If O-linked glycans are not present at all, reaction rates for all amounts of *As* and *As-O* glycoprotein should be similar in value as well as consistently low and/or close to zero.

As shown in Table 1 and Figure 15, for 15 μg *As* fetuin, reaction rates were around 2 pmol NeuAc transferred/min for all three trials, and these values were slightly more than half of the rates observed for the same assays carried out on 30 μg of *As* fetuin (3.59 – 3.77 pmol NeuAc transferred/min). In contrast, however, following treatment to remove O-linked glycans, reaction rates were greatly diminished for both 15 and 30 μg of *As-O* fetuin as compared to their *As* counterparts. For example, for Trial 1, the rate obtained for 15 μg of *As-O* fetuin was 0.16 pmol NeuAc transferred/min, which is 8.83% of the rate found using 15 μg of *As* fetuin for that trial. Moreover, the rate obtained in Trial 1 by using 30 μg of *As-O* fetuin (0.170 pmol NeuAc transferred/min) was also

Form of Acceptor	Trial #	Reaction Rate (pmol NeuAc transferred/min)	
		15 μg Acceptor	30 μg Acceptor
As	1	1.84	3.61
	2	2.10	3.59
	3	1.95	3.77
As-O	1	0.16	0.17
	2	0.05	0.17
	3	0.06	0.16

Table 1. Reaction rates obtained for ST3GalII assays with *As* and *As-O* fetuin. Fifteen and 30 μg of each form of the fetuin acceptor were tested in each assay trial, and results from three trials are shown.

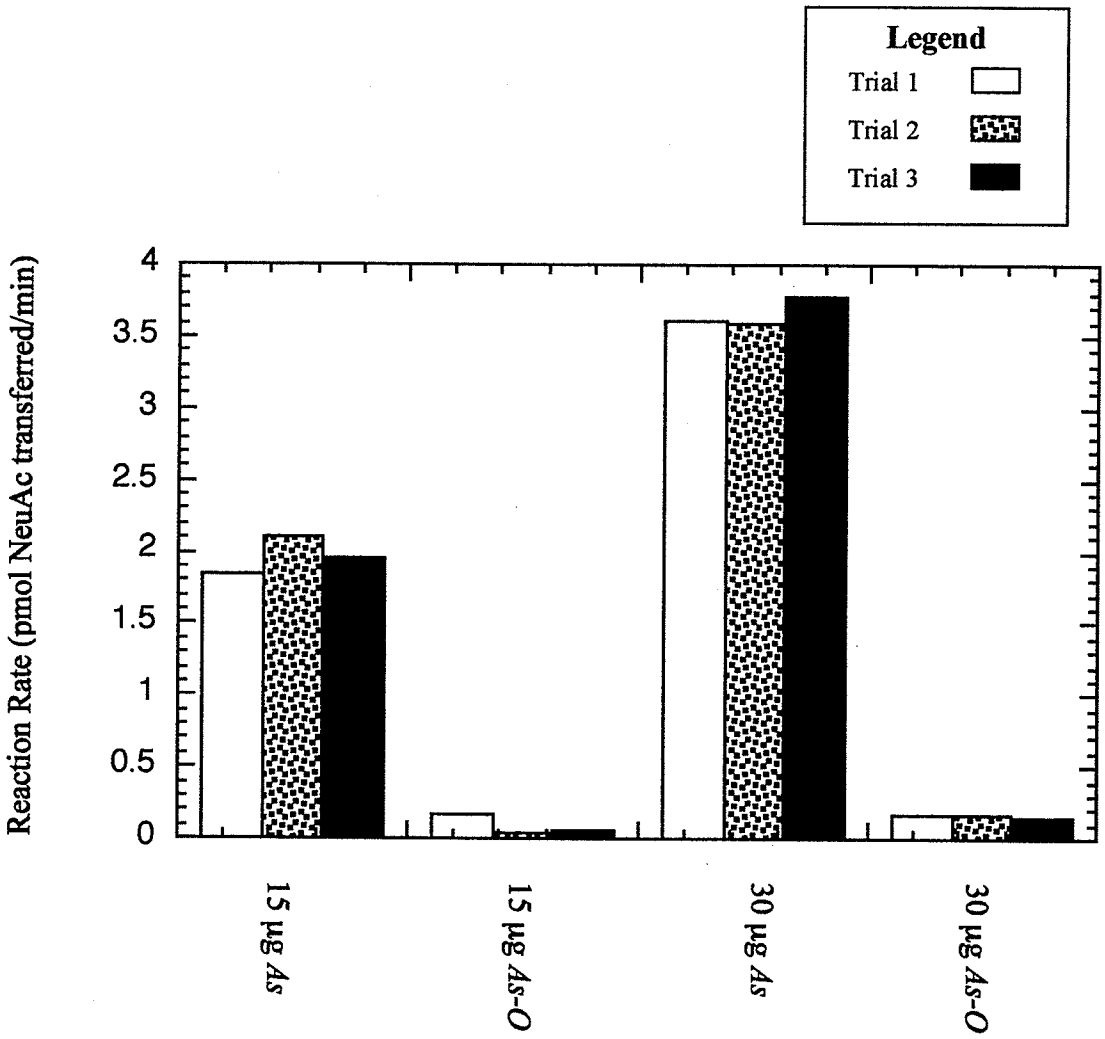

Figure 15. Graphical depiction of results from ST3GalII assays on *As* and *As-O* fetuin. Bars of the same shading type (white, confetti, or black) represent samples assayed in the same trial.

found to be greatly diminished as compared to the assay using the same amount of *As* fetuin (4.7% of the activity).

Interestingly, the results obtained for the assaying of *As/As-O* α_1-AGP with ST3GalII were different. In this case, the reaction rates obtained for all three trials and for both versions and amounts of acceptor were fairly consistent and low, as shown in Table 2 and Figure 16. For instance, for Trial 2, 15 μg of *As* α_1-AGP yielded 0.01 pmol NeuAc transferred/min; whereas 30 μg of the same acceptor, 15 μg of *As-O* α_1-AGP, and 30 μg of *As-O* α_1-AGP yielded 0.04, 0.03, and 0.03 pmol NeuAc transferred/min, respectively. Essentially, the pattern of fairly high ST3GalII reaction rates for both amounts of the *As* fetuin and much lower rates for both amounts the *As-O* fetuin was not observed in the α_1-AGP. In fact, all of the reaction rates for α_1-AGP both prior to and following O-linked chain removal are much more similar to the reaction rates for *As-O* fetuin than they are to those for the *As* fetuin.

Form of Acceptor	Trial #	Reaction Rate (pmol NeuAc transferred/min)	
		15 μg Acceptor	30 μg Acceptor
As	1	0.02	0.02
	2	0.01	0.04
	3	0.01	0.07
As-O	1	0.00	0.07
	2	0.03	0.03
	3	0.02	0.01

Table 2. Reaction rates obtained for ST3GalII assays with *As* and *As-O* α_1-AGP. Fifteen and 30 μg of each form of the α_1-AGP acceptor were tested in each assay trial, and results from three trials are shown.

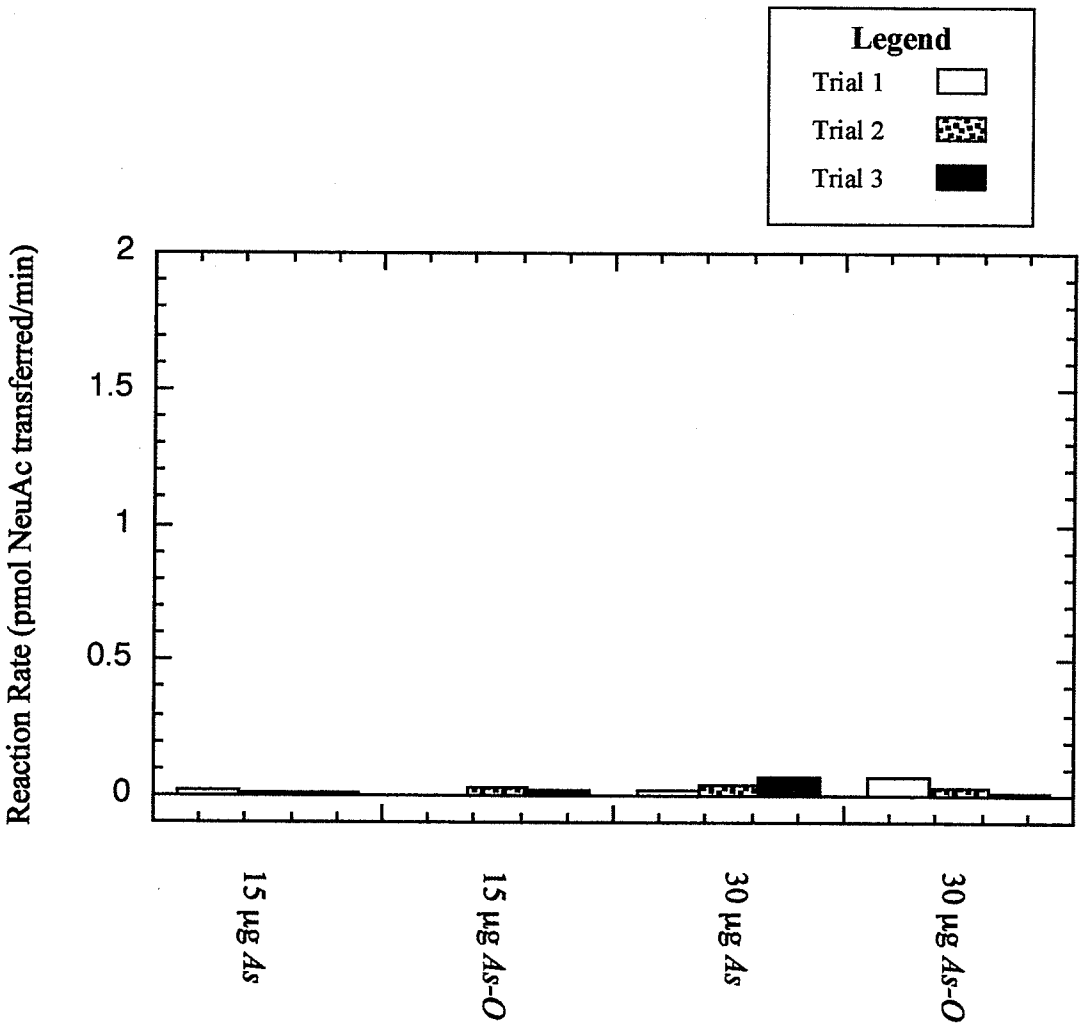

Figure 16. Graphical depiction of results from ST3GalII assays on *As* and *As-O* α_1-AGP. Bars of the same shading type (white, confetti, or black) represent samples assayed in the same trial.

C. Assessment of Removal of N-Linked β1,4-Galactose Residues in Bovine Fetuin and α_1-AGP

To remove N-linked β1,4-Gal residues, the *As-O* forms of bovine fetuin and α_1-AGP (which had been determined to have any O-linked glycans successfully removed based upon results from ST3GalII assays) were treated with a recombinant β1,4-galactosidase from *S. pneumoniae* with a specificity for hydrolysis of N-linked β1,4-Gal residues as outlined in Results. This step is performed to remove any β1,4-Gal structures from the macromolecular acceptor structure while leaving the desired β1,3-Gal residues intact. Most of the N-linked galactose residues found in fetuin have been shown to be in β1,4-linkages to N-acetylglucosamine along with some β1,3-Gal (Townsend *et al.*, 1989), and as with the presence of O-linked carbohydrates, the proportions and types of N-linked galactose residues are unpublished for α_1-AGP. Following treatment to remove β1,4-Gal, the now *-G* versions of both glycoproteins along with their *+G* counterparts (also known as *As-O*) were assayed against commercial ST6GalI and ST3GalIII and reaction rates were determined. ST6GalI attaches sialic acid in a α2,6-linkage onto N-linked β1,4-Gal structures only. Therefore, reaction rates obtained for ST6GalI assays with 30 μg of *+G* fetuin should be about double of those obtained for 15 μg of the same form of fetuin acceptor, and *–G* fetuin, if successfully treated with β1,4-galactosidase, should have reaction rates that are similar for both 15 and 30 μg samples and close to zero and/or negligible when compared to reaction rates for both amounts of *+G* acceptor. All values should be low and close to zero (i.e. negligible) for both forms and amounts of α_1-AGP acceptor if this glycoprotein does not initially contain β1,4-Gal residues. The same reaction pattern described for *+G* and *–G* fetuin should occur in α_1-AGP if the

glycoprotein does initially contain N-linked β1,4-Gal. On the other hand, ST3GalIII attaches sialic acid through an α2,3-linkage onto both β1,4- and β1,3-Gal, with a preference for attachment onto β1,3-Gal (Weinstein *et al.*, 1982). As a result, all of the results of ST3GalIII assays on both *+G* and *–G* forms of acceptor should yield significant reaction rates even if β1,4-Gal is successfully cleaved if the glycans contain β1,3-Gal (as in fetuin), with 15 μg amounts of acceptor yielding reaction rates approximately half of those for the corresponding 30 μg amounts of the same form of acceptor. ST3GalIII reaction rates on the *–G* forms of the glycoprotein acceptors are expected to be somewhat lower than those on the *+G* acceptor forms (when comparing identical amounts of acceptor) as the *+G* form should contain both β1,4- and β1,3-Gal to which α2,3-NeuAc can attach; while the *–G* form should only contain β1,3-Gal. Therefore, reaction of ST3GalIII with *–G* α_1-AGP here as for fetuin can indicate the presence of β1,3-linked galactose residues in bovine α_1-AGP.

As shown in Table 3 and Figure 17, ST6GalI reaction rates for 15 μg of *+G* fetuin were about 50% of the rates acquired for 30 μg of the same forms of acceptor. For instance, for Trial 2, the reaction rate obtained for 15 μg *+G* acceptor was 3.14 pmol NeuAc transferred/min, which is 47.1% of the rate value for 30 μg *+G* sample. Moreover, all *+G* ST6GalI reaction rates were significantly greater then those for all amounts of the *–G* form of fetuin; rates ranged from 1.41 pmol NeuAc transferred/min for 15 μg of *+G* fetuin to 6.67 pmol NeuAc transferred/min for 30 μg of the same acceptor, whereas for *–G* fetuin, rates were between -0.01 pmol NeuAc transferred/min

for 15 μg acceptor and 0.08 pmol NeuAc transferred/min for 30 μg acceptor. For Trial 3, using 30 μg of fetuin acceptor, the *–G* reaction rate of 0.08 pmol NeuAc transferred/min was only 2.3% of the rate achieved using *+G* fetuin.

In Table 4 and Figure 18, for the ST3GalIII assays on *+G* and *–G* fetuin, both amounts of *+G* and *–G* were able to act as acceptors for the ST3GalIII in Trial 1, with the rate of 0.27 pmol NeuAc transferred/min for 15 μg of the *–G* fetuin comprising 41.6% of the rate value obtained for 15 μg *+G* fetuin and the rate of 0.47 pmol NeuAc transferred/min for 30 μg *–G* fetuin comprising 45.3% of the value obtained for the same amount of *+G* fetuin. Furthermore, reaction rates for 15 μg acceptor were close to half those obtained for 30 μg of the same form of acceptor for the same trial (e.g. value for 15 μg of *–G* fetuin was 0.27 pmol NeuAc transferred/min; 58.3% of the rate for double the amount of the same acceptor). However, results for Trials 2 and 3 were somewhat different, as both indicated comparatively high enzyme activity for both amounts of the *+G* fetuin as compared to the *–G* fetuin. For instance, in Trial 3, ST3GalIII reaction rates were 1.04 and 1.86 pmol NeuAc transferred/min for 15 and 30 μg *+G* fetuin, respectively, whereas both values obtained for 15 and 30 μg of *–G* fetuin were essentially negligible at approximately –0.40 pmol NeuAc transferred/min each.

Form of Acceptor	Trial #	Reaction Rate (pmol NeuAc transferred/min)	
		15 μg Acceptor	30 μg Acceptor
+G	1	1.41	2.93
	2	3.14	6.67
	3	1.93	3.67
-G	1	-0.01	-0.01
	2	0.13	-0.02
	3	0.05	0.08

Table 3. Reaction rates obtained for ST6GalI assays with *+G* and *-G* fetuin. Fifteen and 30 μg of each form of the fetuin acceptor were tested in each assay trial, and results from three trials are shown.

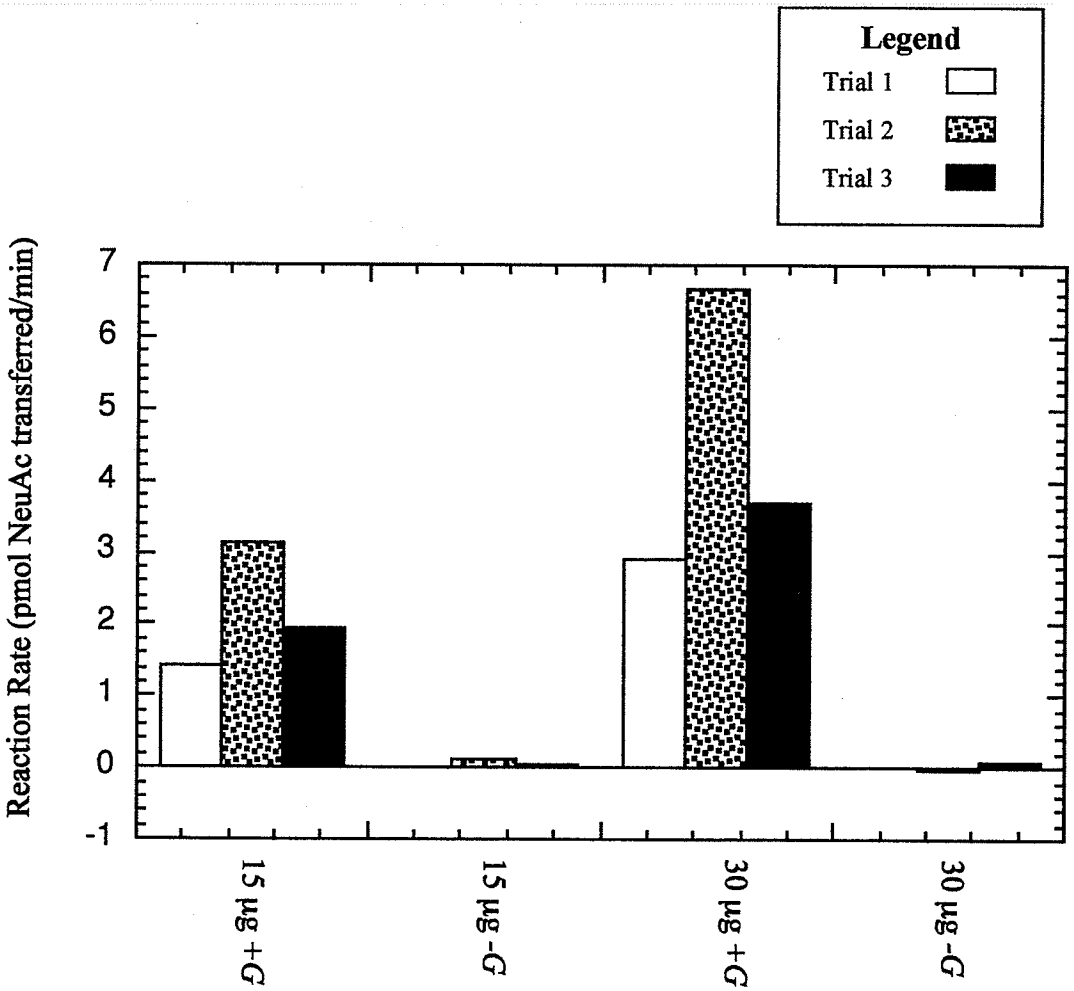

Figure 17. Graphical depiction of results from ST6GalI assays on +*G* and -*G* fetuin. Bars of the same shading type (white, confetti, or black) represent samples assayed in the same trial.

Form of Acceptor	Trial #	Reaction Rate (pmol NeuAc transferred/min)	
		15 μg Acceptor	30 μg Acceptor
+*G*	1	0.66	1.04
	2	0.78	1.33
	3	1.04	1.86
-*G*	1	0.27	0.47
	2	-0.07	-0.15
	3	-0.44	-0.41

Table 4. Reaction rates obtained for ST3GalIII assays with +*G* and -*G* fetuin. Fifteen and 30 μg of each form of the fetuin acceptor were tested in each assay trial, and results from three trials are shown.

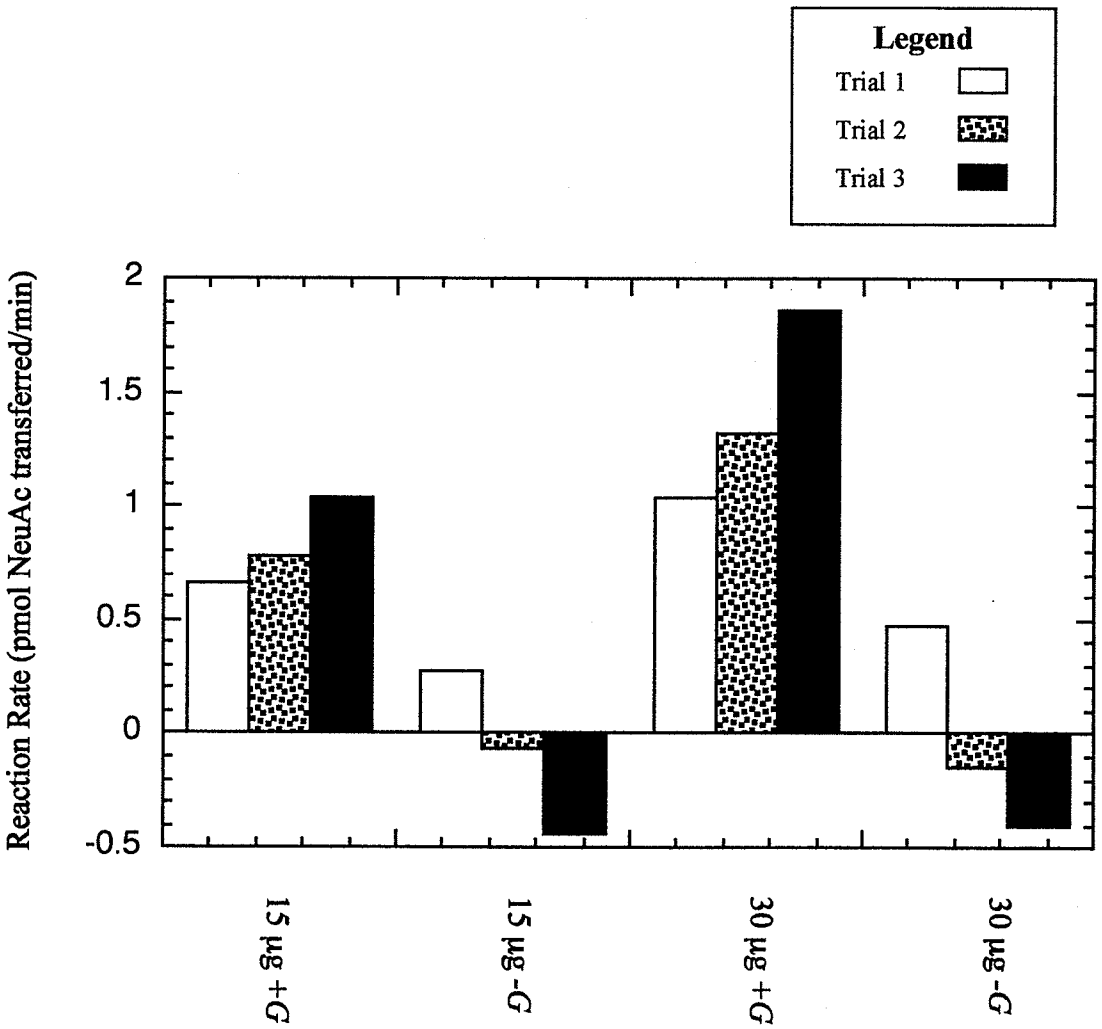

Figure 18. Graphical depiction of results from ST3GalIII assays on +*G* and -*G* fetuin. Bars of the same shading type (white, confetti, or black) represent samples assayed in the same trial.

As with the testing of +*G* fetuin with ST6GalI, reaction rates obtained for this assay using +*G* α_1-AGP were relatively high, with 15 μg acceptor yielding values about half of those obtained for 30 μg acceptor. As shown in Table 5 and Figure 19, for Trial 1, 15 μg of +*G* resulted in a reaction rate of 5.34 pmol NeuAc transferred/min, as compared to 30 μg of the same glycoprotein, whose rate attained was 9.56 pmol NeuAc transferred/min. Following β1,4-galactosidase treatment to hydrolyze β1,4-Gal residues, reaction rates were greatly diminished for the now –*G* α_1-AGP as compared to those obtained for the +*G* α_1-AGP, which was also observed for the fetuin forms. For instance, in Trial 3, 30 μg of –*G* α_1-AGP had a rate value of 0.08 pmol NeuAc transferred/min, which comprised only 1.3% of the reaction rate for 30 μg +*G* fetuin in the same trial. In addition, all amounts of –*G* α_1-AGP resulted in low rates with similar values for both 15 and 30 μg amounts (e.g. for Trial 2, reaction rates were –0.26 and –0.31 pmol NeuAc transferred/min for 15 and 30 μg α_1-AGP, respectively).

The results obtained for the testing of +*G*/-*G* α_1-AGP with ST3GalIII shown in Table 6 and Figure 20 yielded results quite a bit different from those found for the same forms of fetuin. For the α_1-AGP assays, the –*G* acceptor rates were observed to be close to the values of or even higher than the rates reached for the same amounts of +*G* α_1-AGP. As examples, the rate obtained for 15 μg of +*G* α_1-AGP in Trial 2 was 1.42 pmol NeuAc transferred/min while the rate was 1.26 pmol NeuAc transferred/min for the same amount of –*G* α_1-AGP; for Trial 1, the reaction rate of 3.36 pmol NeuAc transferred/min

for 30 μg -*G* α_1-AGP was actually 2.6 times the value obtained for 30 μg of +*G* α_1-AGP. Amounts of acceptor for Trials 2 and 3 and the –*G* values for Trial 1 were found to result in reaction rates for 15 μg of α_1-AGP acceptor that were around 50% of those found for 30 μg of the same form of acceptor, with 15 μg of +*G* α_1-AGP in Trial 3 yielding 53.3% of the reaction rate of 3.03 pmol NeuAc transferred/min observed for 30 μg of the same acceptor, and 15 μg of –*G* α_1-AGP in Trial 1 resulting in 58.5% of the rate value found for 30 μg of –*G* α_1-AGP in the same trial. This was not always the case for Trial 1, however, as the +*G* reaction rates were quite a bit closer in value as the reaction rate for 15 μg +*G* α_1-AGP was 79.2% of the value found for twice the amount of the same acceptor (1.30 pmol NeuAc transferred/min).

Form of Acceptor	Trial #	Reaction Rate (pmol NeuAc transferred/min)	
		15 µg Acceptor	30 µg Acceptor
+G	1	5.34	9.56
	2	2.12	3.93
	3	3.48	6.50
-G	1	-0.18	-0.11
	2	-0.26	-0.31
	3	0.05	0.08

Table 5. Reaction rates obtained for ST6GalI assays with *+G* and *-G* α_1-AGP. Fifteen and 30 µg of each form of the α_1-AGP acceptor were tested in each assay trial, and results from three trials are shown.

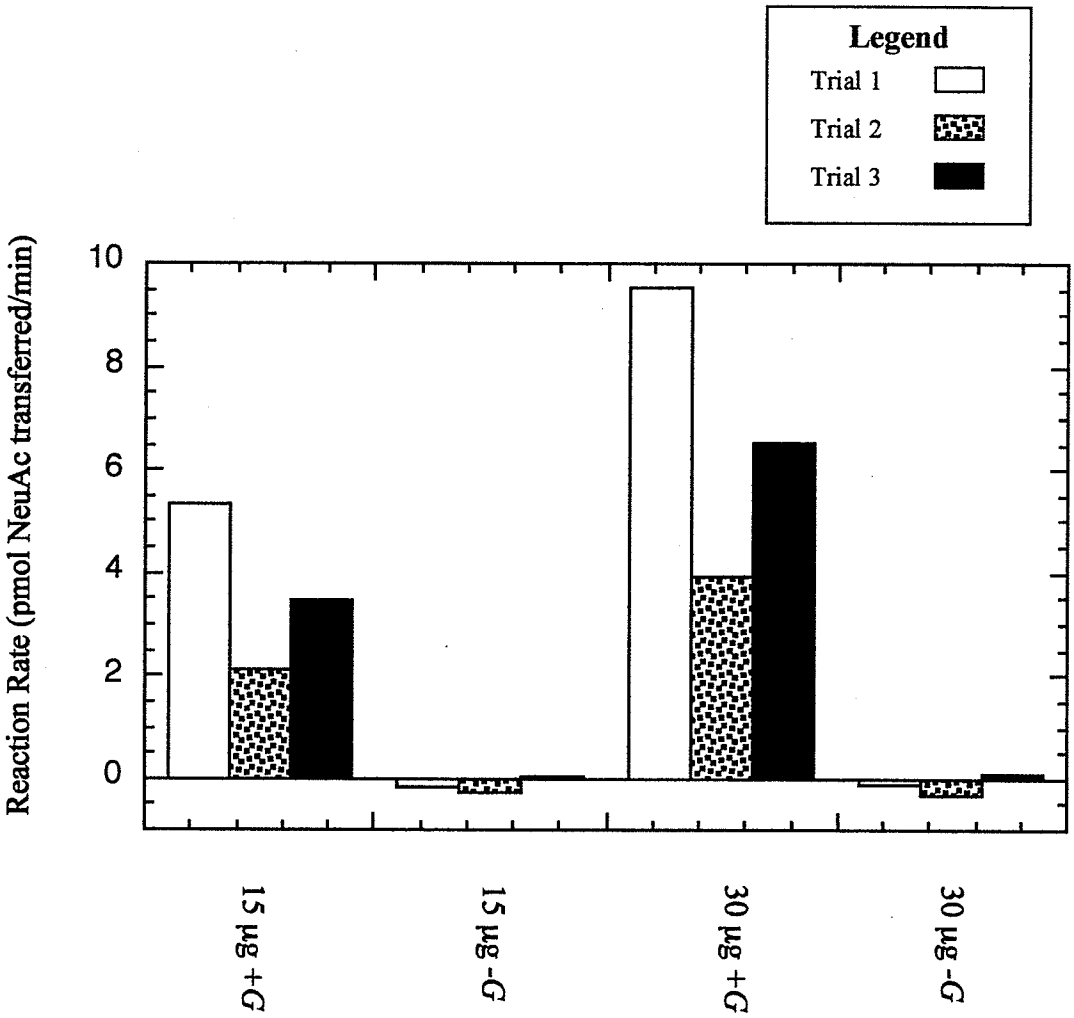

Figure 19. Graphical depiction of results from ST6GalI assays on +*G* and -*G* α_1-AGP. Bars of the same shading type (white, confetti, or black) represent samples assayed in the same trial.

Form of Acceptor	Trial #	Reaction Rate (pmol NeuAc transferred/min)	
		15 μg Acceptor	30 μg Acceptor
+G	1	1.03	1.30
	2	1.42	2.48
	3	1.61	3.03
-G	1	1.97	3.36
	2	1.26	2.83
	3	1.22	3.15

Table 6. Reaction rates obtained for ST3GalIII assays with *+G* and *-G* α_1-AGP. Fifteen and 30 μg of each form of the α_1-AGP acceptor were tested in each assay trial, and results from three trials are shown.

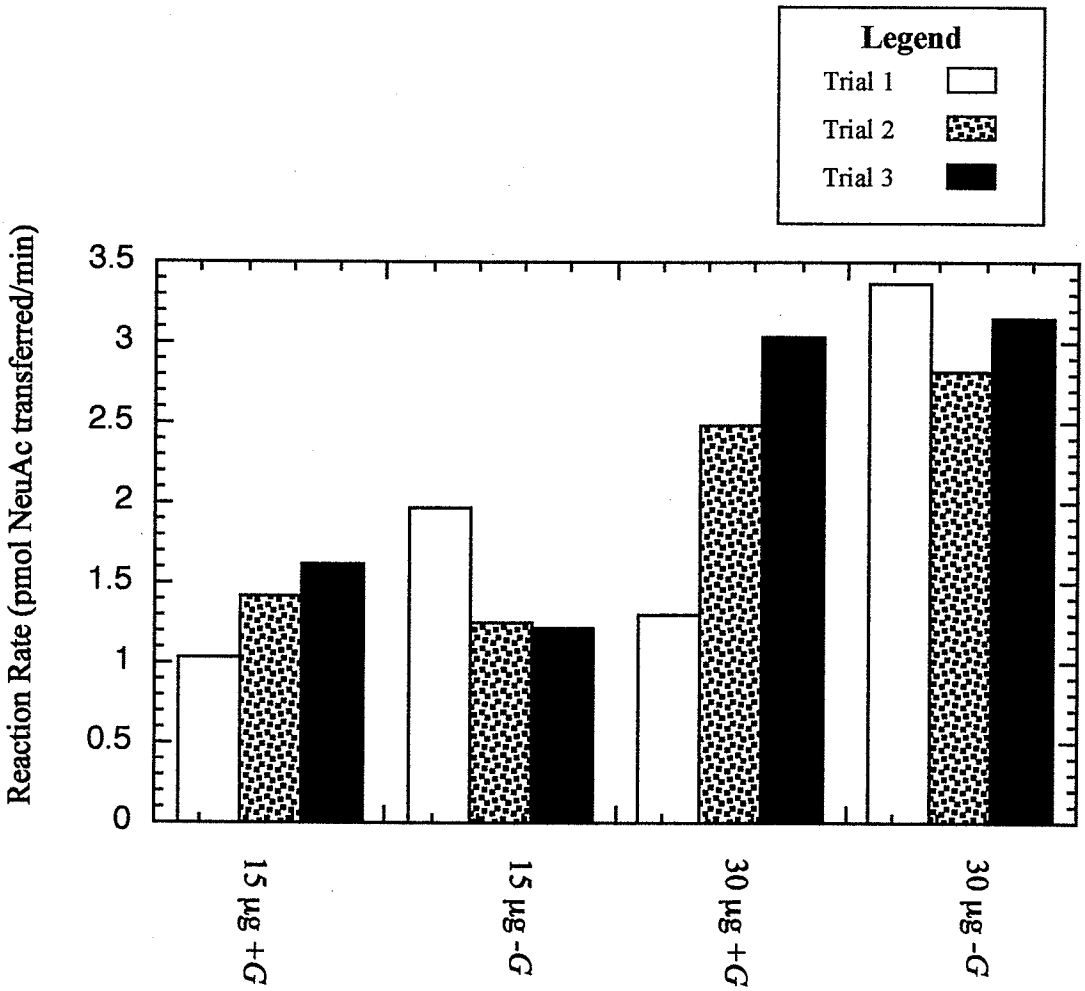

Figure 20. Graphical depiction of results from ST3GalIII assays on +*G* and -*G* α_1-AGP. Bars of the same shading type (white, confetti, or black) represent samples assayed in the same trial.

D. Assessment of Addition of Terminal N-Linked α2,3-Sialic Acid to β1,3-Galactose Residues in Bovine Fetuin and α_1-AGP

Following successful removal of N-linked β1,4-Gal residues, the *–G* (also referred to as *A*) forms of bovine fetuin and α_1-AGP were treated with *S. frugiperda* recombinant forms of rat ST3GalIII along with CMP-NeuAc to catalyze the re-addition of α2,3-linked sialic acid onto the remaining N-linked β1,3-Gal residues of the glycoproteins. This addition of these residues is imperative in the preparation of macromolecular acceptors for ST6GlcNAcI as it has been demonstrated using oligosaccharide acceptors that the synthesis of the disialylated structure NeuAcα2,3Galβ1,3(NeuAcα2,6)GlcNAc requires the sequential addition of sialic acid via the action of two sialyltransferases: firstly, the α2,3-linked terminal sialic acid by ST3GalIII, and secondly, the side-arm α2,6-NeuAc by ST6GlcNAcI (Paulson *et al.*, 1984). Hence, treating the fetuin and α_1-AGP with ST3GalIII and CMP-NeuAc results in the terminal addition of α2,3-NeuAc to the presumed N-linked glycan structure Galβ1,3GlcNAc-R in the glycoproteins, creating the final structure required for ST6GlcNAcI reaction. Moreover, it is vital that the carbohydrates are completely sialylated in this step in the production of macromolecular acceptors as the form of ST6GlcNAcI utilized in the assaying of the final acceptors is impure, i.e. it is contained in a suspension of liver Golgi particles that would likely include other ST's with a potential to react with free N-linked β1,3-Gal residues and result in false positive results for ST6GlcNAcI reaction. Following this re-sialylation treatment, the now-complete glycoprotein acceptors were assayed along with their corresponding *A* forms using the same recombinant ST3GalIII to determine if sialylation was complete in the *S*

glycoproteins. Reaction rates should be significant and reasonably high for 30 μg of the *A* acceptors as these should contain free N-linked β1,3-Gal residues available for reaction (15 μg of the *A* acceptors should yield about half of these rate values). On the other hand, the *S* glycoproteins should yield insignificant reaction with ST3GalIII (approximately at the same level for both 15 and 30 μg of *S* acceptor) when compared to the magnitude of reaction obtained for the *A* acceptors if resialylation is complete. The fetuin structure is known to contain β1,3-linked galactose in several of its N-linked oligosaccharides (Townsend *et al.*, 1989), however, this feature is again unpublished for the α_1-AGP. Consequently, if the above pattern of reaction with ST3GalIII is observed for the testing of *A* and *S* α_1-AGP (similar to that seen in the same forms of fetuin), the presence of these N-linked β1,3-Gal residues in bovine α_1-AGP may be indicated.

As shown in Table 7 and Figure 21, the pattern of high *A*-low *S* reaction rates occurred in Trials 1 and 2 for the testing of the fetuin acceptor with ST3GalIII. For Trial 1, the rate value of 0.30 pmol NeuAc transferred/min for 15 μg of *A* fetuin is 52.2% of the value for 30 μg of the same acceptor. Both rates attained for 15 and 30 μg of the *S* fetuin in the same trial were negligible and low compared to the *A* fetuin reaction rates, at –0.01 and 0.00 pmol NeuAc transferred/min, respectively. As noted, the pattern for Trial 2 values was similar, although here, 15 μg of *A* fetuin at 0.07 pmol NeuAc transferred/min was only 12.7% of the reaction rate obtained for double the amount of *A* fetuin. As for Trial 1, ST3GalIII rate values for both amounts of *S* fetuin in Trial 2 were low and negligible compared to the corresponding *A* values (e.g.

–0.176 pmol NeuAc transferred/min for 30 μg of *S* fetuin). In contrast, Trial 3 yielded reaction rates with negative values for both *A* and *S* fetuin. For instance, 30 μg of *A* fetuin resulted in a reaction rate of -0.15 pmol NeuAc transferred/min, whereas the same amount of *S* fetuin had a rate value of –0.48 pmol NeuAc transferred/min. However, the magnitude of the values obtained for both amounts of *A* fetuin in this trial were higher than the same amounts of their *S* counterparts (e.g. previous example and values for 15 μg acceptor in Trial 3; -0.07 and –0.26 pmol NeuAc transferred/min for *A* and *S* fetuin, respectively). Interestingly, values for 15 μg of either *A* or *S* fetuin were actually approximately 50% higher than their 30 μg counterparts, a finding not visualized in Trials 1 or 2.

Form of Acceptor	Trial #	Reaction Rate (pmol NeuAc transferred/min)	
		15 μg Acceptor	30 μg Acceptor
A	1	0.30	0.58
	2	0.07	0.54
	3	-0.07	-0.15
S	1	-0.01	0.00
	2	-0.16	-0.18
	3	-0.26	-0.48

Table 7. Reaction rates obtained for ST3GalIII assays with *A* and *S* fetuin. Fifteen and 30 μg of each form of the fetuin acceptor were tested in each assay trial, and results from three trials are shown.

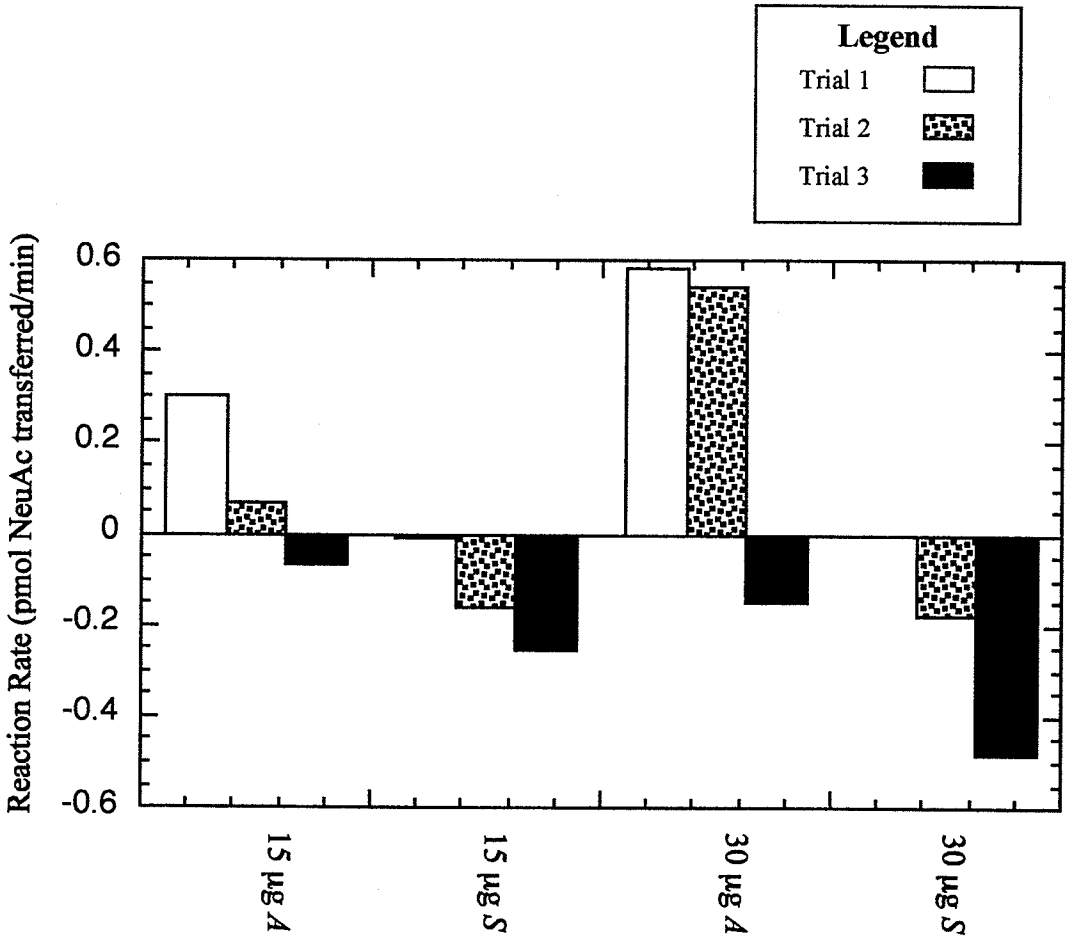

Figure 21. Graphical depiction of results from ST3GalIII assays on *A* and *S* fetuin. Bars of the same shading type (white, confetti, or black) represent samples assayed in the same trial.

For the results of testing of *A* and *S* α_1-AGP with ST3GalIII shown in Table 8 and Figure 22, all three trials yielded reaction rates for *A* α_1-AGP that were significantly higher than those found for *S* α_1-AGP. For example, in Trial 1, 30 μg of *A* α_1-AGP resulted in a rate value of 2.24 pmol NeuAc transferred/min, while the same amount of the corresponding *S* glycoprotein had a value of –0.06 pmol NeuAc transferred/min. Furthermore, 15 μg *A* α_1-AGP reacted with the ST3GalIII about half as much as 30 μg of the same acceptor (e.g. for Trial 2, 1.14 pmol NeuAc transferred/min was obtained for 15 μg of *A* α_1-AGP; 46.6% of the value for twice the amount of acceptor). Rate values were consistently low and negligible for the *S* α_1-AGP in Trials 1 and 3 (e.g. in Trial1, –0.06 pmol NeuAc transferred/min was attained for 15 μg of *S* acceptor as compared to the value for 30 μg of *S* α_1-AGP in this trial given above), but the rate for 15 μg of *S* acceptor in Trial 2 was much lower than that for twice the amount (0.03 pmol NeuAc transferred/min as compared to 0.20 pmol NeuAc transferred/min). Nevertheless, these *S* rate values were, indeed, significantly lower than those found for 15 and 30 μg of *A* α_1-AGP in the same trial shown above.

Form of Acceptor	Trial #	Reaction Rate (pmol NeuAc transferred/min)	
		15 μg Acceptor	30 μg Acceptor
A	1	1.28	2.24
	2	1.41	3.02
	3	0.56	1.14
S	1	-0.06	-0.06
	2	0.03	0.20
	3	0.03	0.00

Table 8. Reaction rates obtained for ST3GalIII assays with *A* and *S* α_1-AGP. Fifteen and 30 μg of each form of the α_1-AGP acceptor were tested in each assay trial, and results from three trials are shown.

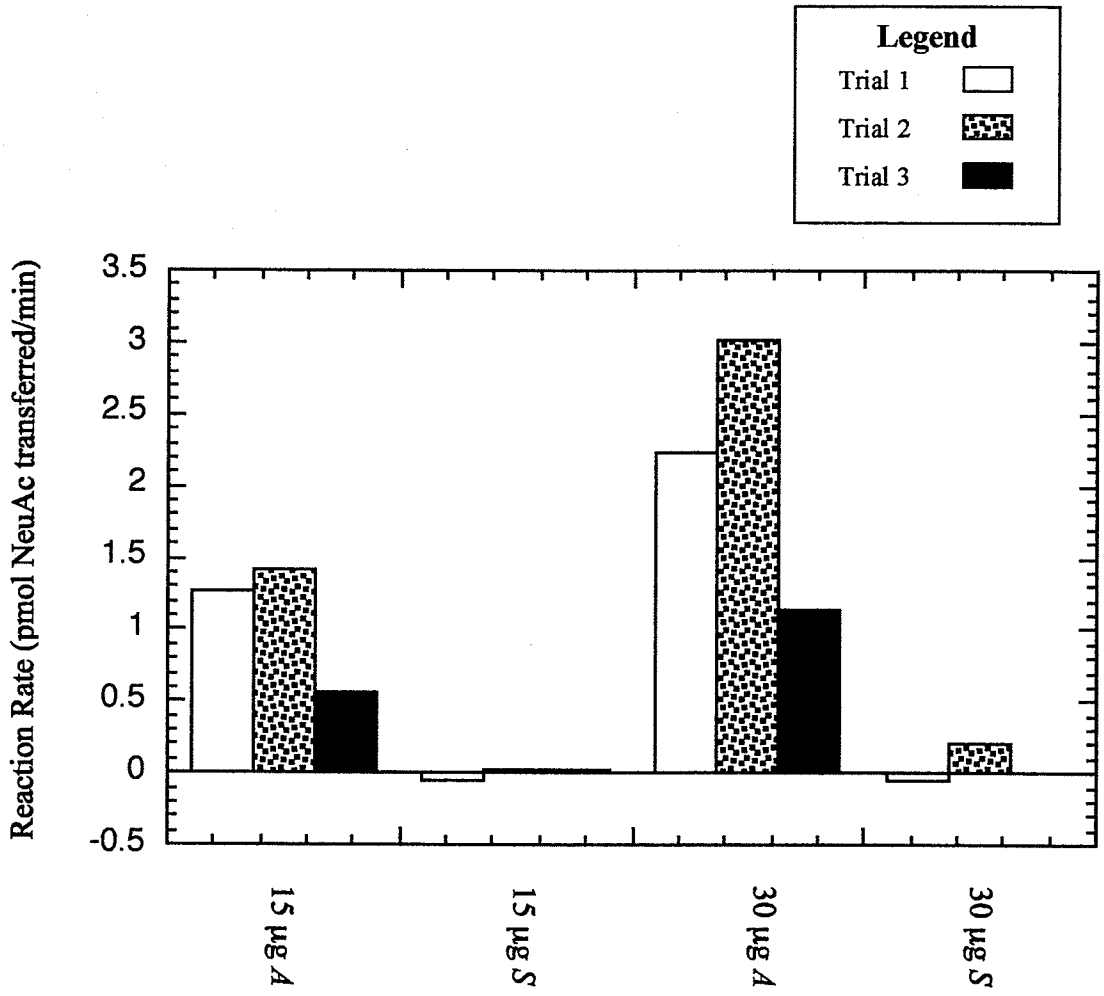

Figure 22. Graphical depiction of results from ST3GalIII assays on *A* and *S* α_1-AGP. Bars of the same shading type (white, confetti, or black) represent samples assayed in the same trial.

E. Assessment of the Ability of Desialylated ST6GlcNAcI Acceptors Prepared from Bovine Fetuin and α_1-AGP to React with ST3GalIII

After the successful completion of the preparation of macromolecular acceptors for ST6GlcNAcI from bovine fetuin and α_1-AGP, a few milligrams of each lyophilized glycoprotein were removed from the total final amount and these samples were again individually subjected to dilute acid hydrolysis at 90°C (as in the first step of ST6GlcNAcI acceptor preparation) to remove the α2,3-linked sialic acid residues added in the resialylation step. This constitutes an essential final step in the preparation of macromolecular acceptors for ST6GlcNAcI as the small now-asialo samples isolated from the final acceptor batches must again have the ability to accept sialic acid from reaction with *S. frugiperda* recombinant rat ST3GalIII in order for the *S* glycoproteins to be used as legitimate acceptors for ST6GlcNAcI. The reason for this is that if the N-linked glycans on the fetuin and α_1-AGP have been reconstructed as expected, the anticipated result is that the asialo- versions of the ST6GlcNAcI acceptors should contain the N-linked disaccharide structure Galβ1,3GlcNAc-R (in effect, the same carbohydrate sequence expected to be found in the *A* versions of the glycoproteins) with the ability to react with ST3GalIII in a predictable fashion. Therefore, the asialo- ST6GlcNAcI fetuin and α_1-AGP acceptors produced here (termed *DS*) were tested along with corresponding amounts of the *S* versions of the glycoproteins for ST3GalIII reaction. If acceptor preparation was successful, it is expected that reaction for either starting glycoprotein should be negligible when compared to *DS* reaction and/or close to zero for both amounts of the *S* forms, while reaction rates should be significantly higher for 15 and 30 μg of the

DS acceptors, with 30 μg *DS* acceptor resulting in a rate value about twice that achieved for half the amount of the same acceptor.

As shown in Table 9 and Figure 23, both trials of the reaction of ST3GalIII with *S* and *DS* fetuin show negligible reaction for both 15 and 30 μg of the *S* fetuin. In fact, all results here displayed negative values for reaction rate ranging from –0.34 pmol NeuAc transferred/min for 15 μg of the *S* form in Trial 1 to –0.95 pmol NeuAc transferred/min in Trial 2 for 30 μg of the same acceptor. In contrast, reaction rates for the *DS* fetuin in both trials were significantly higher than their corresponding *S* values although some of these values were also found to be negative. For instance, 30 μg of the *DS* form in Trial 1 yielded a rate value of –0.09 pmol NeuAc transferred/min whereas in Trial 2, the rate was found to be 0.19 pmol NeuAc transferred/min. However, reaction of ST3GalIII with 15 μg of *DS* fetuin was not found to produce rates at about 50% of the magnitude as with 30 μg of the same acceptor in either trial (e.g. in Trial 1, the rate value for 15 μg of *DS* fetuin at –0.02 pmol NeuAc transferred/min was actually higher than the value for 30 μg of the same acceptor shown above).

Form of Acceptor	Trial #	Reaction Rate (pmol NeuAc transferred/min)	
		15 μg Acceptor	30 μg Acceptor
S	1	-0.34	-0.37
	2	-0.84	-0.95
DS	1	-0.02	-0.09
	2	-0.01	0.19

Table 9. Reaction rates obtained for ST3GalIII assays with *S* and *DS* fetuin. Fifteen and 30 μg of each form of the fetuin acceptor were tested in each assay trial, and results from two trials are shown.

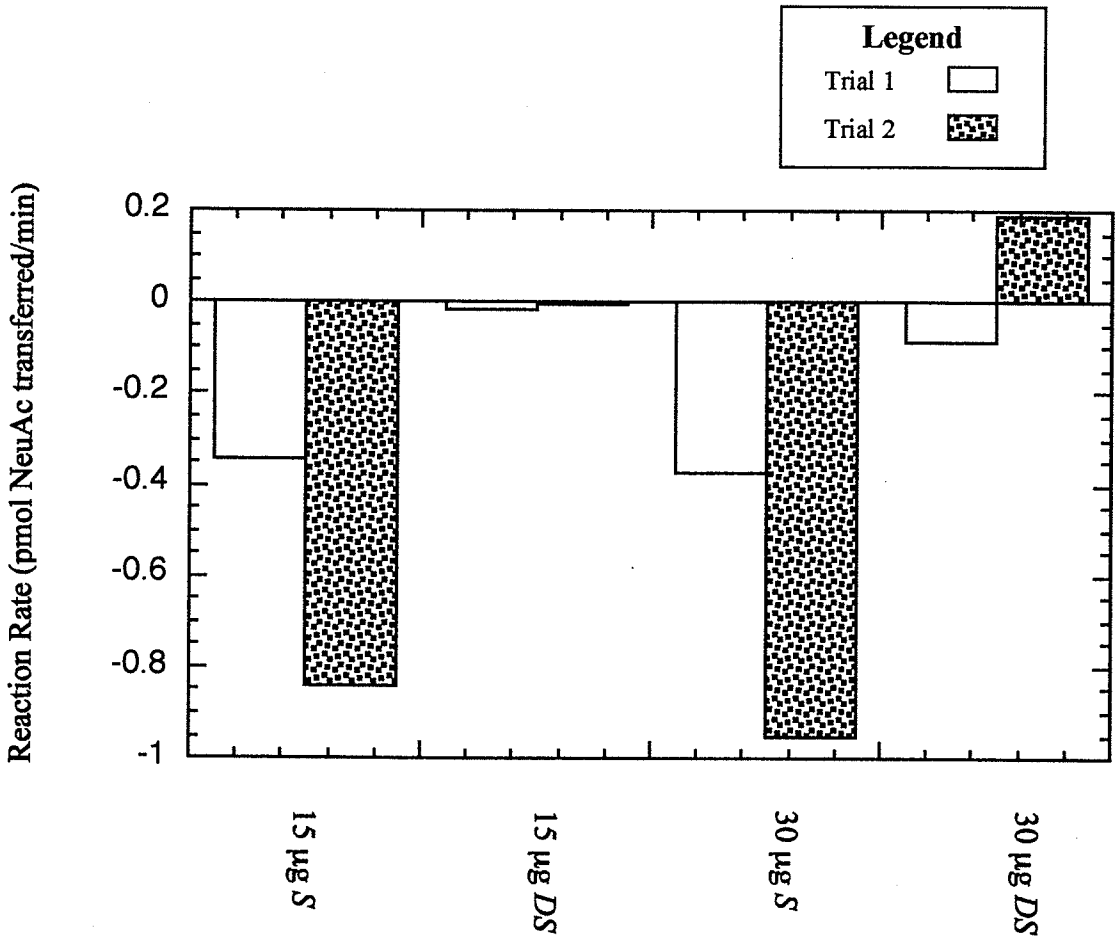

Figure 23. Graphical depiction of results from ST3GalIII assays on *S* and *DS* fetuin. Bars of the same shading type (white or confetti) represent samples assayed in the same trial.

For the testing of *S/DS* α_1-AGP with ST3GalIII, it is evident from the results shown in Table 10 and Figure 24 that reaction rates obtained for *S* α_1-AGP are considerably lower than those of corresponding amounts of *DS* α_1-AGP in both trials. For example, in Trial 1, 30 μg of the *S* form resulted in a rate value of only 8.4% of that obtained for the same amount of *DS* α_1-AGP (the rate of which was 1.34 pmol NeuAc transferred/min). Furthermore, all *S* reaction rates were found to be around the same value and low for both trials, with Trial 2 giving rate values of 0.19 pmol NeuAc transferred/min for 15 μg of *S* α_1-AGP and 0.26 pmol NeuAc transferred/min for twice the amount of the same acceptor. Also in both trials, reaction rates for 15 μg of the *DS* forms of acceptor were actually determined to be greater than 50% of the magnitude of the rate values observed for 30 μg of *DS* α_1-AGP (e.g. in Trial 2, 15 μg of the *DS* α_1-AGP had a rate that was 69.1% of the value found for double the amount of the same acceptor).

Form of Acceptor	Trial #	Reaction Rate (pmol NeuAc transferred/min)	
		15 μg Acceptor	30 μg Acceptor
S	1	-0.08	0.11
	2	0.19	0.26
DS	1	0.82	1.34
	2	1.10	1.59

Table 10. Reaction rates obtained for ST3GalIII assays with *S* and *DS* α_1-AGP. Fifteen and 30 μg of each form of the α_1-AGP acceptor were tested in each assay trial, and results from two trials are shown.

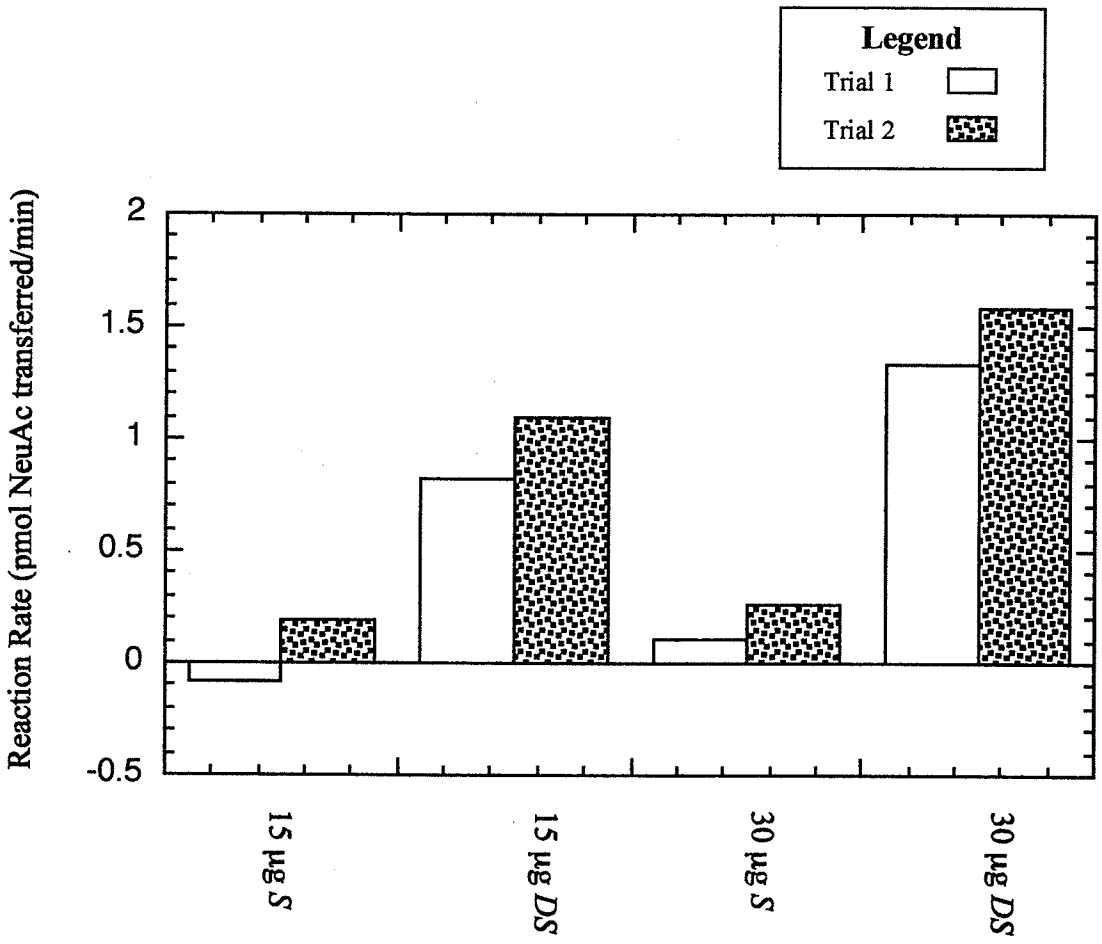

Figure 24. Graphical depiction of results from ST3GalIII assays on *S* and *DS* α_1-AGP. Bars of the same shading type (white or confetti) represent samples assayed in the same trial.

F. Determination of K_m and V_{max} Values for ST6GlcNAcI using Completed Bovine Fetuin and α_1-AGP Macromolecular Acceptors

Once the bovine fetuin and α_1-AGP acceptors had been synthesized according to the sequential procedure given in Methods and all testing assays with commercially available ST's signified the likelihood of having the predicted glycan structure, the acceptors were ready to be assayed with ST6GlcNAcI to determine reaction kinetics. Rat liver Golgi fractions presumed to contain ST6GlcNAcI were prepared using the methods of Leelavathi *et al.* (1970) as modified by Moreman and Touster (1986). The final Golgi preparations were brought to a final concentration of 10 mg/mL in 0.25 M sucrose in potassium phosphate buffer, pH 7.4, and this suspension was used as the enzyme source for assaying the macromolecular glycoprotein acceptors. Various concentrations of both the fetuin and α_1-AGP acceptors were used in these assays, with the fetuin concentrations ranging from 0.007 mM to 0.040 mM (presuming a MW of fetuin of 42,500 Da after modification) and the α_1-AGP was used at concentrations of 0.016 mM to 0.096 mM (presuming a MW of 39,000 Da) in the 80 μL samples containing all the necessary reaction components. Lower concentrations of fetuin were used as this acceptor was found to be soluble in 1 M cacodylate buffer, pH 6.0, only up to a concentration of 4.6 mg/mL, whereas the α_1-AGP acceptor readily dissolved in the same buffer at the concentration of 10 mg/mL that was employed here. High enough amounts of the α_1-AGP acceptor were utilized such that approximately two times K_m could be reached at the highest concentrations used (assuming reaction kinetics for ST6GlcNAcI are similar to those for ST3GalIII) but concentrations of the fetuin acceptor may not have been high enough to reach V_{max} (owing to its low solubility). Amounts of CMP-NeuAc were

assumed to be in excess, and samples were incubated and quantified as given in Methods. Michaelis-Menten kinetic plots were then computer-generated and K_m and V_{max} values were calculated by interpolation from the plots. The rat liver ST6GlcNAcI was predicted to exhibit typical Michaelis-Menten kinetics with both macromolecular acceptors, with linearly increasing reaction rates up to the highest concentrations of either fetuin or α_1-AGP acceptor used at which point the graph would be expected to plateau (i.e. maximum rate reached), allowing for the determination of K_m and V_{max} values.

The fetuin ST6GlcNAcI acceptor did react with the presumed ST6GlcNAcI in the Golgi suspension, producing a kinetic graph with somewhat linearly increasing reaction rate values (first order reaction) that did not appear to have a distinct plateau (refer to Table 11 for the data and Figure 25 for the graph). However, the data did generate a line that was very slightly curved with a tendency towards a plateau (zero order reaction) at the highest concentrations of the fetuin acceptor (0.034 and 0.040 mM acceptor; reaction rates were 1.32 and 1.34 pmol NeuAc transferred/min, respectively). V_{max} and K_m values were attainable from this graph, although these values had high error due to the lack of a distinct plateau. The K_m value obtained was 0.071 mM ($\pm$ 0.017 or 23.4% error), and V_{max} was also obtained at 3.86 pmol NeuAc transferred/min ($\pm$ 1.28 or 33.1% error) (refer to Table 13).

[Acceptor] (mM)	Average Reaction Rate (pmol NeuAc transferred/min)
0.007	0.32 ± 0.02
0.013	0.57 ± 0.02
0.020	0.89 ± 0.01
0.027	1.03 ± 0.02
0.034	1.32 ± 0.03
0.040	1.34 ± 0.12

Table 11. Reaction rates obtained for fetuin acceptor assay with rat liver Golgi ST6GlcNAcI. The experiment utilized rat liver Golgi suspension prepared from the livers of two control rats, and each concentration of fetuin acceptor was assayed three times in the same trial. The average rates obtained are shown here, along with their standard error.

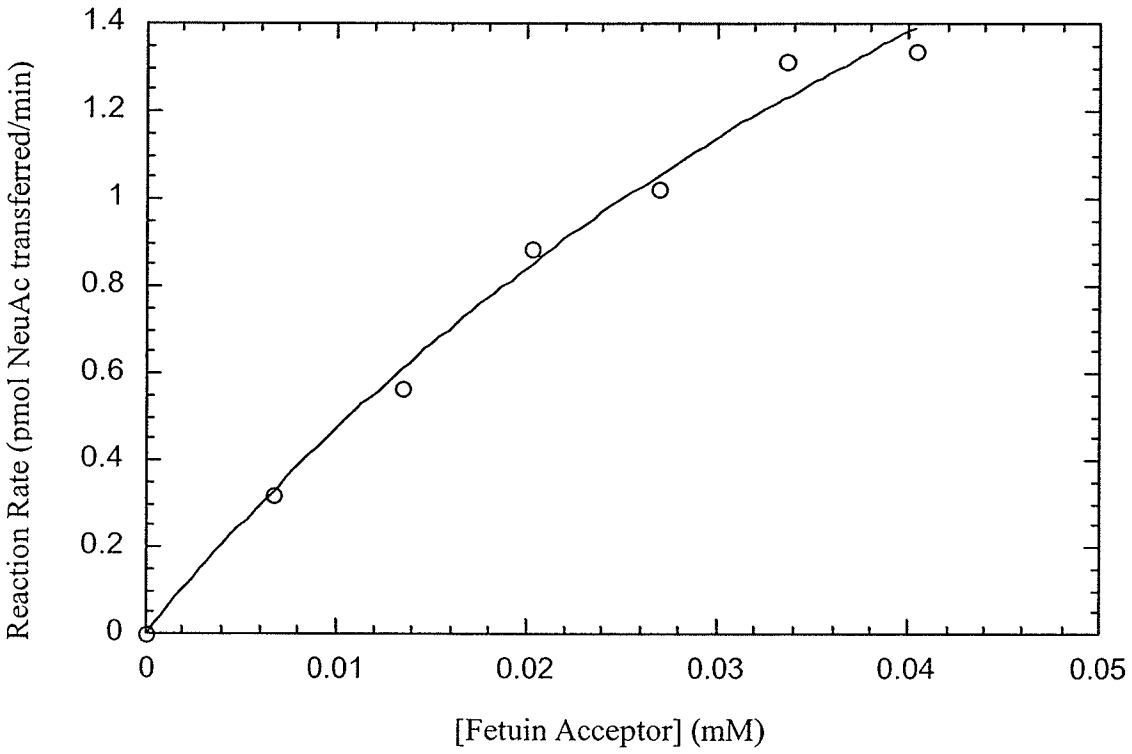

Figure 25. Michaelis-Menten plot obtained for fetuin acceptor assay with rat liver Golgi ST6GlcNAcI. The assay was carried out using Golgi suspension prepared from two rat livers, and each data point represents the average of three samples.

The data from the rat liver Golgi ST6GlcNAcI assay of the completed α_1-AGP acceptor produced a Michaelis-Menten plot somewhat different than that obtained for the fetuin acceptor (see Table 12 for data and Figure 26 for graph). Like the graph for the fetuin acceptor, the values here increased fairly linearly from zero to about 0.032 mM α_1-AGP (which produced a rate of 0.28 pmol NeuAc transferred/min). In contrast to the fetuin acceptor, however, a more distinct region of close to-zero order reaction was observed here, most notably from 0.064 mM to 0.096 mM α_1-AGP acceptor, where the rates only ranged between 0.31 – 0.37 pmol NeuAc transferred/min. In addition, rates attained by ST6GlcNAcI reaction with the α_1-AGP acceptor were lower than those found for fetuin (rate values were between 0.16 and 0.37 pmol NeuAc transferred/min for this acceptor as compared to values from 0.32 to 1.34 pmol NeuAc transferred/min for the fetuin acceptor). K_m and V_{max} values were also determined for the reaction with α_1-AGP, and these were observed to have lower error than those for fetuin (α_1-AGP K_m was 0.029 mM at $\pm$ 0.003 or 8.9% error; V_{max} was 0.47 pmol NeuAc transferred/min at $\pm$ 0.08 or 17.5% error) (see Table 13).

[Acceptor] (mM)	Average Reaction Rate (pmol NeuAc transferred/min)
0.016	0.16 ± 0.04
0.032	0.28 ± 0.05
0.048	0.26 ± 0.07
0.064	0.31 ± 0.10
0.080	0.33 ± 0.10
0.096	0.37 ± 0.01

Table 12. Reaction rates obtained for α_1-AGP acceptor assay with rat liver Golgi ST6GlcNAcI. The experiment utilized rat liver Golgi suspension prepared from the livers of two control rats, and each concentration of α_1-AGP acceptor was assayed three times in the same trial. The average rates obtained are shown here, along with their standard error.

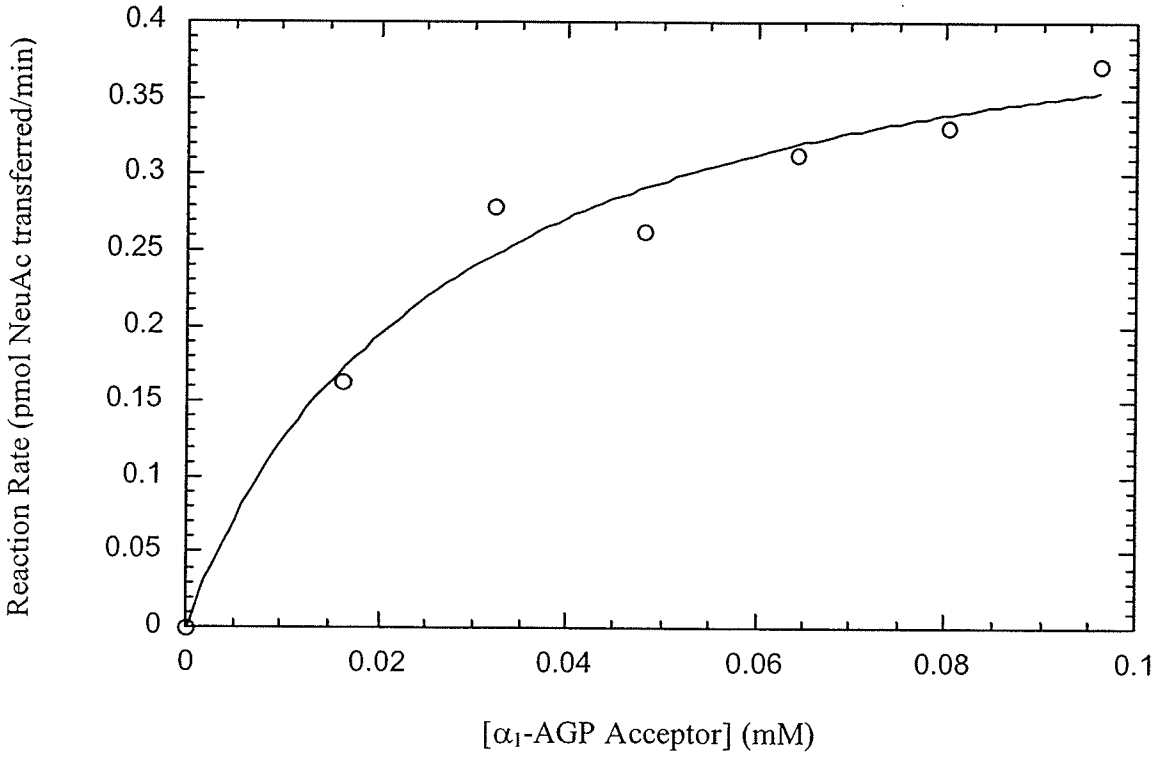

Figure 26. Michaelis-Menten plot obtained for α_1-AGP acceptor assay with rat liver Golgi ST6GlcNAcI. The assay was carried out using Golgi suspension prepared from two rat livers, and each data point represents the average of three samples.

ST6GlcNAcI Acceptor	K_m (mM)	V_{max} (pmol NeuAc transferred/min)
Fetuin	0.071 ± 0.017	3.86 ± 1.28
α_1-AGP	0.029 ± 0.003	0.47 ± 0.08

Table 13. ST6GlcNAcI K_m and V_{max} values determined using bovine fetuin and α_1-AGP macromolecular acceptors.

DISCUSSION

A. Evidence for Linearity of Commercial Sialyltransferase Reaction at Forty-Five Minutes Incubation

To ensure that the ST reaction rates calculated for the macromolecular acceptor testing assays are kinetically valid, the commercial ST's used for the testing (ST3GalII, ST3GalIII, and ST6GalI) were each assayed with a reactive form of the fetuin acceptor varying the time of incubation. For the reaction rates to be considered legitimate, the 45-minute incubation time that was used for all the acceptor testing assays must fall within the linear or first-order portion of a time versus assay response curve, before the zero-order portion is reached. As shown in Figures 12, 13, and 14, the 45-minute time point appears to fall within the linear portion of the response curves just before the plateau. The calculated reaction rates were therefore taken to be valid for all fetuin acceptor testing assays. Time response assays were not performed on the α_1-AGP acceptor as this glycoprotein was mainly studied for investigational purposes as there is no hard scientific data on α_1-AGP's glycan composition. However, α_1-AGP was predicted to have similar kinetics to fetuin with commercial ST3GalIII and ST6GalI and thus reaction rates calculated for the α_1-AGP assays were assumed to be legitimate. Furthermore, the rat liver Golgi ST6GlcNAcI was not tested for reaction linearity with either macromolecular acceptor, but both the completed fetuin and α_1-AGP acceptors yielded Michaelis-Menten plots in ST6GlcNAcI assays after 45 minutes incubation from which K_m and V_{max} could be determined (see Results), and as a result, the reaction kinetics were taken to be valid.

B. Evidence of Hydrolysis of O-Linked Glycans in Bovine Fetuin and α_1-AGP

The first step in preparing macromolecular acceptors for ST6GlcNAcI from bovine fetuin and α_1-AGP entailed treating the desialylated glycoproteins with a modified version of alkaline-borohydride hydrolysis (Likhosherstov *et al.*, 1990) to specifically cleave O-linked carbohydrate chains. The complete removal of O-glycans from the glycoprotein acceptors is essential as the Golgi preparation utilized as a source for ST6GlcNAcI is enzymatically impure, leading to the possibility that O-linked carbohydrates in the acceptor structure could react with O-glycan-specific ST's in the preparation, resulting in false positive results for ST6GlcNAcI reaction with the assay system used. Hence, a valid method to test for the presence or absence of O-glycans in the acceptor structures was required. Initially, the *As* and the *As-O* versions of the fetuin acceptor were run on SDS-PAGE gels so that the molecular weight difference between the two acceptor forms (corresponding to the molecular weight of the O-linked chains) could be visualized, but the *As-O* fetuin was found to run aberrantly in the gels (SDS-PAGE data not presented). Cleaving the O-glycans enzymatically from the fetuin using a commercially purchased O-glycanase instead of the chemical method appeared to solve this problem, but the amount of the glycanase required to treat large amounts of glycoprotein was financially unfeasible and the molecular weight difference between the *As* and *As-O* forms was small and difficult to observe in the gels. Instead, a different sort of enzymatic approach was taken. To test for the removal of O-linked glycans, the *As* and *As-O* glycoproteins were assayed with commercial ST3GalII, a ST with a strict specificity for the Galβ1,3GalNAc sequence on O-linked carbohydrates and negligible reaction with N-linked carbohydrates (Lee *et al.*, 1994). Consequently, when assaying a

glycoprotein with the O-glycans successfully cleaved, the ST3GalII activity should be greatly diminished when compared to the assay results obtained with the same glycoprotein with intact O-linked carbohydrates. In contrast, if a glycoprotein lacks O-chains to begin with, assay results with ST3GalII should be consistent and low for both the *As* and *As-O* forms of the glycoprotein.

The results obtained on ST3GalII reaction with fetuin indicate that the O-glycans were likely successfully cleaved from the protein backbone by alkaline-borohydride hydrolysis (e.g. for 30 μg of fetuin acceptor, the *As* fetuin was found to have only 4.7% of the ST3GalII activity determined for the *As-O* fetuin). Seeing as how the expected results were obtained, this suggests that ST3GalII assaying of fetuin likely denotes a valid method to test for the absence of O-glycans.

For the bovine α_1-AGP, the carbohydrate makeup in terms of the presence or absence of O-linked carbohydrates has not been published. Data obtained here with the ST3GalII assays shows that the bovine α_1-AGP structure likely does not contain O-linked carbohydrates to begin with as all rates for ST3GalII reaction ranged from 0.01 – 0.04 pmol NeuAc transferred/minute for both *As* and *As-O* forms with no large difference in activity noted between the different forms in contrast to the results found for fetuin, which is known to contain three O-glycans (Spiro and Bhoyroo, 1974). These results strongly indicate that treating and testing the α_1-AGP for the removal of O-glycans is unnecessary and was henceforth left out when preparing ST6GlcNAcI acceptors from this glycoprotein.

C. Evidence of Hydrolysis of N-Linked β1,4-Galactose Residues in Bovine Fetuin and α_1-AGP

Following removal of O-glycans in fetuin and desialylation in bovine α_1-AGP, the *+G* forms of both potential acceptors were treated with *S. pneumoniae* β1,4-galactosidase to cleave N-linked β1,4-Gal residues from the structures which could potentially react with certain Golgi ST enzymes during ST6GlcNAcI assays. To test whether or not N-linked β1,4-Gal removal was complete, the *–G* glycoproteins were assayed alongside their *+G* counterparts with commercial ST6GalI and ST3GalIII. ST6GalI reacts specifically with N-linked β1,4-Gal and thus ST6GalI reaction was used as the main confirmatory test for β1,4-Gal hydrolysis, while ST3GalIII reacts with both N-linked β1,3- and β1,4-Gal (with a preference for β1,3-Gal) (Weinstein *et al.*, 1982) and reaction of the acceptors with this enzyme was used as additional supporting evidence. Moreover, reacting *+G* and *-G* α_1-AGP with ST3GalIII (after β1,4-linked Gal residues are determined to have been removed successfully by virtue of ST6GalI assays) can also serve as a putative test to determine the presence or absence of β1,3-Gal in this glycoprotein. This feature is required in the acceptor structure for ST6GlcNAcI reaction.

The data obtained on assaying the *+G* and *-G* fetuin and α_1-AGP acceptors with ST6GalI demonstrates that it is probable that the β1,4-galactosidase treatment results in sufficient removal of β1,4-Gal residues (e.g. for 30 μg of acceptor, the reaction rates attained for *–G* α_1-AGP and fetuin ranged from 1.3 – 2.3% of the values determined for the *+G* versions). As the expected results were obtained for this assay, ST6GalI testing

of fetuin and α_1-AGP acceptors appears to be an effective testing method to examine β1,4-Gal hydrolysis.

Assays of the fetuin acceptor with ST3GalIII produced conflicting results for the different trials, indicating that this particular test may not be very useful in the determination of N-linked β1,4-Gal removal for fetuin. It is expected that reaction rates should generally be higher for the *+G* acceptors as compared to the *–G* versions, considering that the *+G* forms contain both β1,3- and β1,4-Gal with a potential to react with this particular ST, although significant reaction should occur with the β1,3-Gal. The fetuin appeared to react in this fashion with the ST3GalIII for Trial 1, with the reaction rates for the *–G* forms being approximately 40% of those attained by the same amounts of *+G* fetuin. However, in the other two trials, reaction of the *–G* fetuin acceptors with the enzyme actually produced negative rates (ranging from -0.41 to -0.07 pmol NeuAc transferred/minute) while the *+G* fetuin reacted significantly (0.66 to 1.86 pmol NeuAc transferred/minute). This may have been due to possible inaccessibility of the ST3GalIII to the few β1,3-Gal residues in the fetuin structure resulting from denaturation of the protein by the dilute acid and alkaline-borohydride hydrolysis treatments. N-linked β1,4-Gal residues are much more plentiful than β1,3-Gal in fetuin (Townsend *et al.,* 1989) and thus the magnitude of the ST3GalIII reaction in the *+G* fetuin in Trials 2 and 3 may arise from the fact that denaturation of the protein backbone may still leave enough of the abundant β1,4-linked Gal residues exposed for reaction.

On the other hand, the *+G* and *–G* α_1-AGP did react more or less as expected with the ST3GalIII with both amounts of the *–G* α_1-AGP reacting significantly for all trials as compared to the *+G* values (rates ranged from 1.22 – 3.36 pmol NeuAc transferred/minute for the *–G* α_1-AGP and from 1.03 – 3.03 pmol NeuAc transferred/minute for the *+G* form). The effect of negligible ST3GalIII reaction with the *–G* form as noted with the fetuin in two trials was not observed with the α_1-AGP. This could be due to the fact that the α_1-AGP may not have been as severely denatured as the fetuin since the α_1-AGP did not undergo alkaline-borohydride hydrolysis and was noted to maintain a high solubility in cacodylate buffer even after glycan modification treatments, whereas fetuin often precipitated out. This lack of severe denaturation may have allowed access of the ST3GalIII to more putative reactive Gal residues in the α_1-AGP structure. Furthermore, it was observed that a greater magnitude of reaction actually occurred with the *–G* α_1-AGP as compared to the same amount of the *+G* form in some cases (e.g. in Trial 1, 30 μg of *–G* α_1-AGP produced a value 258% of the corresponding value for the *+G* α_1-AGP). This may be explained by the preference of the ST3GalIII enzyme for β1,3-Gal residues over β1,4-Gal; the lack of β1,4-Gal in the *–G* form of α_1-AGP could result in greater accessibility of the ST to β1,3-Gal residues resulting in a greater degree of reaction than that observed in the *+G* form. As demonstrated by this data on ST3GalIII assays, α_1-AGP likely does contain the required Galβ1,3GlcNAc N-linkage for acceptor preparation due to the observed significant reaction of the glycoprotein with this enzyme after β1,4-galactosidase treatment, and the pattern of predictable results attained in assays with ST3GalIII suggest that it may serve

as a useful test to aid in confirming the absence of β1,4-Gal sugars in the *–G* form of α_1-AGP.

D. Evidence of Formation of the Structure NeuAcα2,3Galβ1,3GlcNAc-R in Bovine Fetuin and α_1-AGP

To create the precursor N-linked glycan structure NeuAcα2,3Galβ1,3GlcNAc-R required for ST6GlcNAcI reaction, the *–G(A)* forms of bovine fetuin and α_1-AGP were incubated with a recombinant form of rat ST3GalIII along with CMP-NeuAc to catalyze the re-addition of α2,3-linked NeuAc onto the terminal β1,3-Gal residues. To determine if the sialylation reaction was complete, the *A* and *S* forms of each glycoprotein were assayed with the same ST with negligible reaction of the enzyme with the *S* acceptors (as compared to the *A* forms) being indicative of complete sialylation. Once again, testing the acceptors here was essential as free β1,3-Gal residues in the final structure would be capable of reacting with ST enzymes other than ST6GlcNAcI present in the Golgi suspension used later on.

For both the fetuin and the α_1-AGP, ST3GalIII testing of the resialylated *S* form appears to be a valid method to confirm the likelihood of complete sialylation. For the fetuin, all *A* reaction rates were significantly higher than their *S* counterparts, as expected (e.g. for 30 μg of acceptor in Trial 1, the *A* form produced a reaction rate of 0.58 pmol NeuAc transferred/minute whereas the *S* form's rate was 0.00 pmol NeuAc transferred/minute). Interestingly, several values, particularly in Trial 3 (where they ranged from –0.48 to –0.07 pmol NeuAc transferred/minute) were negative, which indicates that the reaction rates obtained for samples with no acceptor present were actually higher than those found for samples with acceptor. Speculatively, this may have been due to the non-specific precipitation of the ^{14}C-labelled CMP-NeuAc in the control

(no acceptor) samples, leading to false positive ST3GalIII results higher than those found in the *A* and *S* samples. This is an unexpected result without a clearly defined explanation that will require future experimentation to determine the cause. Nevertheless, the magnitude of the *A* rate values in Trial 3 were all still larger than those determined for the *S* form even though they were all negative, indicating that the assay most likely still worked, with the completed fetuin acceptor containing the proper glycan sequence. This certainly suggests that false positive reaction may have occurred in the no acceptor samples with the samples with acceptor reacting as expected. Conversely, the α_1-AGP reacted in a very predictable way with the ST3GalIII, with all reaction rates for the *A* forms being appreciably higher than the values for the *S* forms (e.g. in Trial 2, for 15 μg of acceptor, the *S* rate was only about 2% of the rate determined for the same amount of *A* α_1-AGP). As both the *A* and *S* versions of the acceptor glycoproteins used reacted in a predictable and relatively consistent fashion when tested with ST3GalIII following treatment to catalyze the addition of α2,3-NeuAc to the carbohydrate structure, it may be assumed that the methods used to create the new linkage and to assay for its presence are likely valid.

Following putative confirmation of the presence of the NeuAcα2,3Galβ1,3GlcNAc-R structure in the bovine fetuin and α_1-AGP by recombinant rat ST3GalIII assays, small amounts of the now-complete ST6GlcNAcI acceptors were desialylated by dilute acid hydrolysis. The now-*DS* acceptors were assayed alongside the *S* forms with the ST3GalIII again because if the *DS* form contains the carbohydrate structure assumed to have been created during the acceptor preparation (specifically, N-

linked Galβ1,3GlcNAc-R), it should react significantly with this particular ST whereas the *S* form should exhibit little or no reaction. This step was used as an additional confirmatory test for the presence of the NeuAcα2,3Galβ1,3GlcNAc-R oligosaccharide in the acceptor structure.

For the ST3GalIII assays of the *S* and *DS* glycoproteins, both the fetuin and α_1-AGP displayed rate values similar to the predicted results. All the rates attained for the *S* forms were significantly lower than those determined for corresponding amounts of *DS* acceptors (e.g. for 30 μg of the acceptors in Trial 2, the *DS* form of fetuin reacted to an extent of 0.19 pmol NeuAc transferred/minute whereas the corresponding *S* form's rate was only –0.95 pmol NeuAc transferred/minute; for the α_1-AGP, the magnitude of the *DS* reaction was more than six times that of the *S* reaction). These results indicate that the structures expected to be created during the ST6GlcNAcI acceptor preparation procedure, namely Galβ1,3GlcNAc-R and NeuAcα2,3Galβ1,3GlcNAc-R, likely do exist in the *DS* and *S* acceptor forms respectively, and that the *S* form contains the required glycan structure to react specifically with ST6GlcNAcI from a rat liver Golgi preparation with little chance of reaction occurring with other ST's in the mixture.

E. Evaluation of ST6GlcNAcI Kinetics with Completed Bovine Fetuin and α_1-AGP Acceptors

Following the completion of the fetuin and α_1-AGP acceptors and confirmation of the predicted glycan structure in each glycoprotein by the various ST testing assays, the acceptors were assayed for ST6GlcNAcI activity using a rat liver Golgi suspension as the enzyme source. Kinetic graphs were then plotted using the data obtained, and K_m and V_{max} values were determined for ST6GlcNAcI reaction with each macromolecular acceptor.

Although K_m and V_{max} values were able to be calculated for the fetuin ST6GlcNAcI acceptor, the Michaelis-Menten plot did not show evidence of a distinct plateau required to determine K_m and V_{max} and hence the values that were obtained for these parameters had relatively high error (K_m: 0.071 mM with 23.4% error; V_{max}: 3.86 pmol NeuAc transferred/minute with 33.1% error). Therefore, these results cannot be taken as accurate for K_m and V_{max} determinations for ST6GlcNAcI with this particular macromolecular acceptor. This is likely due to the fact that the fetuin acceptor was only sparingly soluble in the 1 M cacodylate buffer, pH 6.0, used as a solvent, and as a result, could only be brought up to a concentration of 4.6 mg/mL before precipitation occurred (as compared to the α_1-AGP acceptor, which easily dissolved at 10 mg/mL). The lack of solubility of the fetuin may have been caused by severe denaturation of the protein backbone in the molecule, possibly originating from the modified alkaline-borohydride treatment utilized to remove O-linked carbohydrates, an extremely harsh procedure considering the high pH, the temperature (60°C), and the length of incubation (16 hours).

Denaturation of the protein could have also been facilitated by the dilute acid hydrolysis used to hydrolyze NeuAc residues, although this treatment did not appear to affect the solubility of the α_1-AGP. This denaturation may have prevented the actual V_{max} for the fetuin acceptor with ST6GlcNAcI from actually being reached (which can be visualized by the lack of a clear zero-order portion on the Michaelis-Menten plot, Figure 22). Consequently, the K_m and V_{max} values determined here may be overestimates of the true values due to the difficulty in gauging the level at which the Michaelis-Menten plot plateaus since it is not clearly evident. Also, the concentration at which K_m was determined to be was higher than any of the fetuin acceptor concentrations utilized, adding to the error in estimating a value. These difficulties indicate that the use of fetuin modified as described herein is not a good prospect as a substrate for ST6GlcNAcI.

In contrast, the α_1-AGP yielded a Michaelis-Menten plot for ST6GlcNAcI reaction with a more distinct region of zero-order reaction following the linear portion, allowing for the determination of K_m and V_{max} with significantly lower error than for fetuin (for α_1-AGP, K_m: 0.029 mM with 8.9% error; V_{max}: 0.47 pmol NeuAc transferred/minute with 17.5% error). Few problems were encountered in dissolving the α_1-AGP acceptor as were found with fetuin; in fact, the α_1-AGP appeared to solubilize well at a concentration of 10 mg/mL in 1 M cacodylate buffer, the level estimated to approximate 2 x K_m. This may have been due to the fact that the denaturing alkaline-borohydride treatment was not used in the preparation of the α_1-AGP acceptor. As a result, the higher concentrations of this acceptor used in the ST6GlcNAcI assays (as opposed to fetuin) allowed for the more accurate determination of K_m as this value fell

within the range of concentrations tested. On the other hand, as the Michaelis-Menten plot did not completely plateau, the V_{max} value was not quite reached with the array of α_1-AGP acceptor concentrations tested and rather was estimated based upon the curvature of the plot in the plateau region and the value determined for K_m. Consequently, the V_{max} obtained here for ST6GlcNAcI reaction with α_1-AGP may not be accurate. To prevent this, constructing a Michaelis-Menten plot with more data points and/or using higher concentrations of α_1-AGP so that both K_m and V_{max} fall within the plot may allow for more accurate determinations of both parameters.

Although the K_m and V_{max} values determined for ST6GlcNAcI reaction with the macromolecular acceptors prepared from bovine fetuin and α_1-AGP differ somewhat in value from one acceptor to the next and most were calculated to have a considerable amount of error in their determinations (as high as 33.1%), both acceptors appear to have reacted as expected in ST6GlcNAcI assays (each acceptor produced a Michaelis-Menten plot with a tendency towards the expected shape from which K_m and V_{max} values for ST6GlcNAcI reaction could be elucidated). Considering that this was the case, the preparation of macromolecular acceptors for ST6GlcNAcI from fetuin and α_1-AGP appears to have been successful.

F. Conclusions and Future Prospects

In conclusion, these results indicate that the primary goal of this thesis, to successfully produce macromolecular acceptors for the little-studied ST, ST6GlcNAcI, has been accomplished. Moreover, an innovative method of creating the acceptors through a series of chemical and enzymatic "glycan-refashioning" steps has been introduced, along with a procedure to test for the presence of the proper carbohydrate structure at each step, allowing for a much more rapid qualification of the acceptors during their production as compared to traditional analytical methods. And furthermore, information has been obtained on the glycan structure of the poorly characterized bovine α_1-AGP, specifically that it appears to lack O-linked glycans and likely contains N-linked β1,3-Gal residues.

Although these preliminary studies have shown that it is indeed possible to create ST-specific macromolecular acceptors from existing glycoproteins without specific glycan analysis following each modification of the carbohydrate structure, structural analysis of the altered carbohydrates by analytical methods like HPAEC-PAD, NMR, and/or mass spectrometry is still required to further qualify the methods used. In other words, it cannot be stated with complete certainty that the NeuAcα2,3Galβ1,3GlcNAc-R structure was actually created in the fetuin or α_1-AGP until specific analytical data demonstrates that this is the case, although the precise reaction (or lack of reaction) of the acceptors during the various stages in preparation with highly-specific recombinant ST's indicate that the expected structure has probably been created. However, explicit structural data would shed light onto the actual sugar linkages present in the acceptors

and whether or not contaminating linkages incapable of reacting with the recombinant ST's used as "testing" enzymes yet capable of reacting with other ST's in the rat liver Golgi suspension used for ST6GlcNAcI occur at all in the final acceptor structures. And furthermore, while the recombinant ST assays have indicated previously unpublished glycan structural features of bovine α_1-AGP, again, it is impossible to ascertain if this is actually the case without sound structural information to back up the enzyme assay results. Only after the proposed structural features are confirmed by these methods can the kinetic data on ST6GlcNAcI reaction with α_1-AGP be validated.

As noted in the previous section, unlike the α_1-AGP, it was very difficult to solubilize the completed fetuin acceptor which was attributed to possible denaturation of the protein backbone by the modified Carlson degradation procedure used to hydrolyze O-glycans. As a result, the ST6GlcNAcI K_m and V_{max} could not be reached with the range of fetuin concentrations used and thus the values were estimated with fairly high error (23.4 – 33.1%). To circumvent this problem in future studies, removing O-linked glycans from the fetuin through a milder treatment such as the use of a commercial O-glycanase instead of the alkaline-borohydride hydrolysis may be necessary to prevent denaturation and the resulting solubility problems with this acceptor which may allow for determination of kinetic values for ST6GlcNAcI reaction with lower error. This would increase fetuin's viability for use as a potential acceptor for ST6GlcNAcI or other ST enzymes.

As indicated in the Introduction, when assaying GT enzymes, macromolecular acceptors are the closest approximations of the naturally occurring acceptors present *in vivo*, and hence they can provide kinetics similar to what would be found within eukaryotic systems. Accordingly, the K_m and V_{max} data obtained for ST6GlcNAcI reaction with the fetuin and α_1-AGP acceptors should be closer to *in vivo* kinetics than if the oligosaccharide LST_a had been used as an acceptor. However, the structures of the macromolecular acceptors created here are not exactly like actual glycoproteins found *in vivo*. Firstly, the protein moieties of both the fetuin and α_1-AGP have lost their three dimensional structure due to denaturing treatments in their preparations, with the fetuin denaturation being the most drastic. Secondly, the removal of certain carbohydrates from the glycoproteins (such as O-linked glycans and β1,4-linked Gal) was essential to prevent contaminating ST cross-reaction during rat liver Golgi ST6GlcNAcI assays. Conversely, in natural systems, these residues would be intact in the acceptors and they would be expected to be terminally sialylated by the time they reach the terminal Golgi saccules where ST6GlcNAcI is probably located (considering that this ST would catalyze the final NeuAc addition to the glycoproteins). The lack of these features in the macromolecular acceptors created *in vitro* could potentially have a drastic effect on enzyme kinetics due to altered access of the ST6GlcNAcI to the site of reaction as compared to the *in vivo* acceptors. To counteract some of these effects, different steps could be taking when preparing the acceptors in the laboratory. To begin with, as mentioned earlier, denaturation of the protein backbone may be minimized by utilizing enzymatic rather than chemical means to hydrolyze NeuAc residues and O-glycans from the acceptor glycoproteins. In addition, following completion of the acceptors (*S* forms), the

hydrolyzed monosaccharide residues could be re-added to the structures sequentially via recombinant enzymes to replicate the actual glycan structure of the acceptors *in vivo*. However, re-adding the sugars to the glycoproteins and testing for their presence would be laborious and time-consuming, and GT's required to catalyze all the necessary linkages may be unavailable in pure form. And furthermore, having these sugar linkages present in the acceptor structure may have a negligible effect on ST6GlcNAcI kinetics. Nevertheless, maintaining three dimensional structure in the acceptor proteins is likely to have a larger effect on ST6GlcNAcI reaction and this is something that should be investigated in future preparations. However, it should also be noted that widely accepted methods for the preparation of acceptors for ST assays involve treatments just as harsh as those utilized in this study.

Despite the difficulties encountered in preparing bovine fetuin and α_1-AGP macromolecular acceptors for ST6GlcNAcI and the fact that the procedure should be further qualified by direct analysis of the acceptor glycans, this method still affords an easy, rapid technique for preparing ST acceptors from readily accessible glycoproteins. Moreover, this procedure also shows promise for the production of acceptors from fetuin and α_1-AGP for ST enzymes other than ST6GlcNAcI; ongoing work in this laboratory has involved studying reaction kinetics of the ST's ST3GalIII and ST6GalI on specific macromolecular acceptors prepared from these glycoproteins (unpublished data).

Regarding ST6GlcNAcI itself, the next step in its study should be to assay the enzyme with the macromolecular acceptors alongside the oligosaccharide LST_a under

identical conditions so a direct comparison can be made of oligosaccharide versus macromolecular acceptor reaction kinetics. The most suitable macromolecular acceptor for this based on these studies would be the bovine α_1-AGP. LST_a assays were actually attempted here, but the assays gave indeterminate results from which K_m and V_{max} values could not be calculated (data not shown). In addition, the two previous studies on ST6GlcNAcI did not include determinations for K_m and V_{max} from LST_a reaction to which the results in this study could be directly compared (Paulson *et al.*, 1984; de Heij *et al.*, 1986). This data is essential so the effect of having the reactive oligosaccharide NeuAcα2,3Galβ1,3GlcNAc bound onto a protein can be effectively gauged. In addition, ST6GlcNAcI's ability to act as an acute phase reactant should be examined. Increased activity of a different ST, ST6GalI, has been observed following inflammation in rat liver (Kaplan *et al.*, 1983) and in the serum of patients suffering from chronic renal failure (Thorne-Tjomsland *et al.*, 2000). Another previous study indicated that levels of ST6GlcNAcI (along with the ST's ST3GalIII and SAT-1) appear to be altered during the acute phase response, as well, with activity of the rat Golgi versions of the enzymes decreasing about 50% 24 to 36 hours post-inflammation before recovering (Richardson, 1998). A similar pattern of decreased ST6GlcNAcI activity was noted for kidney and spleen versions of the enzyme (Richardson, 1998). Moreover, a slight decline in ST6GlcNAcI activity was observed 36 hours post-inflammation when rat serum versions of the ST were assayed with LST_a (Richardson, 1998). In contrast to this result, however, no significant activity was detected in the present study when serum samples from renal transplant patients were assayed for ST6GlcNAcI activity using the fetuin macromolecular acceptor (data not shown). Furthermore, control serum also yielded

negligible ST6GlcNAcI reaction rates with the same acceptor, indicating that a catalytically active form of this enzyme may not be secreted into the serum of humans even following inflammation. Assays of the same acceptor with control rat serum also resulted in extremely low reaction rates (data not shown). The discrepancy in these serum ST6GlcNAcI assay results may have occurred due to altered reactivity of the enzyme with LST_a as compared to the macromolecular fetuin acceptor, or they may simply be due to a different distribution of the ST in the rat as opposed to the human. It is possible that ST6GlcNAcI may be restricted primarily to organs and tissues in the human such as the liver and placenta as previously noted (de Heij *et al.*, 1986) with little secretion into the serum. Clearly, this is one area of study on ST6GlcNAcI that deserves future consideration as some interesting (and potentially clinically useful) findings could result.

The long-term goal for ST6GlcNAcI study involves the purification and eventual cloning of the enzyme. Several attempts have been made by Paulson and associates to purify the enzyme from rat liver Golgi, but the ST's labile nature has thus far prevented it from being purified to homogeneity (unpublished data). However, even if purification of ST6GlcNAcI does prove to be impossible, production of recombinant forms of the enzyme by expression cloning (which does not require prior purification of the GT) may be achievable. Pure forms of ST6GlcNAcI would allow for more in-depth analysis of the enzyme's reactions kinetics to be conducted since pure ST preparations are generally more stable than less pure forms (like rat liver Golgi fractions). Also, macromolecular acceptors could be prepared simply by the desialylation and subsequent α2,3-NeuAc addition to glycoproteins that contain the required Galβ1,3GlcNAc-R structure as cross-

reaction of the acceptors with other ST's would not be an issue if a pure suspension of ST6GlcNAcI could be utilized. This was the approach taken in a comparison of K_m and V_{max} values with native and recombinant ST's (Williams *et al.*, 1995). The significance of the side arm α2,6-NeuAc added by this enzyme could then be analyzed if this method was employed. Only then can the importance of ST6GlcNAcI be fully realized.

One of the main intents of this investigation into the poorly-studied ST enzyme ST6GlcNAcI has been to highlight the importance of understanding and characterizing the reactions catalyzed by one of the many different GT enzymes. It is solely through the reactions of these enzymes that the wealth of carbohydrate containing compounds be synthesized as many of these glycoconjugates have been determined to have important functions *in vivo* with the glycan portions often comprising the main functional elements. Accordingly, it is now realized that the significance of carbohydrates is at very least tantamount to that of protein and DNA, as the lack of proper glycan complement results in serious diseases like CDG or more common disorders such as osteoarthritis.
As research in the field of glycobiology progresses into the new century, hopefully many of the mechanisms underlying the significance of these glycan structures will be elucidated and practical applications of the findings may eventually be used clinically.

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