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Review Article

Asparagine-Linked Glycosylation: Specificity and Function of Oligosaccharyl Transferase

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Contents

1. Introduction 1565
2. Asparagine-Linked Glycosylation 1567
3. Biochemical Characterization of Oligosaccharyl Transferase 1567
4. Substrates for N-Linked Glycosylation 1569
   4.1 Lipid-linked oligosaccharide donor 1569
   4.2 Peptide acceptor 1569
5. Conformational Requirements 1570
6. Mechanistic Considerations 1572
7. Conformational Consequences of Co-translational Protein Glycosylation 1575
8. Conclusions 1576

1. Introduction

Glycosylation is potentially the most complex category of protein modification reactions within eukaryotic systems.[1] An incredible degree of diversity is introduced into glycoproteins by the wide array of monosaccharides available as well as the potential for different chemical linkages between each pair of carbohydrates.[2] The size of each oligosaccharide chain in glycoproteins can vary greatly, ranging from a single monosaccharide to complex, branched structures comprising as many as 15–40 sugars. In addition, many proteins are glycosylated at multiple sites with different carbohydrate groups; more than 20 terminal oligosaccharide sequences are available and can be used in combination to generate further diversity.[3] In many cases, this structural diversity defines the biological role of each of the modified proteins. Glycoproteins have been implicated in such varied processes as the immune response,[4] proper intracellular targeting,[5] intercellular recognition,[6] and protein folding, stability and solubility.[7–10] These functions are often modulated by the structure of both the oligosaccharide and the protein. An understanding of the biosynthesis of glycoproteins is of intense interest because of the diversity displayed by critical biomolecules.

Carbohydrate modifications of proteins fall into three general categories: N-linked modification of asparagine, O-linked modification of serine or threonine and glycosyl-phosphatidyl inositol derivatization of the C-terminus carboxyl group (Fig. 1).[11] Each of these transformations is catalyzed by one or more enzymes which demonstrate different peptide sequence requirements and reaction specificities. N-Linked glycosylation is catalyzed by a single enzyme, oligosaccharyl transferase (OT), and involves the co-translational transfer of a lipid-linked tetradecasaccharide ($GlcNAc_2$-Man_9-Glc_3) to an asparagine side chain within a nascent polypeptide. The subsequent diversification of these conjugates arises from enzyme catalyzed processing steps that occur in the endoplasmic reticulum (ER) and Golgi apparatus after the addition of the first triantennary oligosaccharide complex. In contrast, O-linked glycosylation proceeds through the direct, post-translational transfer of single monosaccharides to threonine and/or serine residues within folded polypeptides. In general, the peptide sequence specificities of the O-linked glycosylation enzymes are not well understood[12] and activity is attributed to several different enzymes. For example, the principal mode of O-linked

Abbreviations: Ac, acetyl; Aib, α-aminoisobutyric acid; Amb, γ-aminobutyrine; Asn(γS), thioasparagine; BMTS, *N*-biotinoyl(aminoethane)thiolsulfonate; Boc, *t*-butoxycarbonyl; Bz, benzoyl; Cbz, carbobenzyloxy; CD, circular dichroism; Dns, dansyl; Dol, dolichol; Dol-P, dolichol phosphate; Dol-PP, dolichol pyrophosphate; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; FET, fluorescence energy transfer; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GPI, glycosyl phosphatidyl inositol; isoAsn, isoasparagine; Man, mannose; MMTS, methyl methanethiolsulfonate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancements; Orn, ornithine; OT, oligosaccharyl transferase; PC, phosphatidylcholine; UDP–GalNAc, uridine diphosphate-*N*-acetylgalactosamine.

Samsung Bioepis Exhibit 1091
Page 1

glycosylation in yeast involves the transfer of a single mannose from the lipid-linked donor dolichol phosphate mannose (Dol-P-Man) to a hydroxy amino acid,[13] while one example of O-linked glycosylation in mammalian systems involves the transfer of an *N*-acetylgalactosamine residue from uridine diphosphate-*N*-acetylgalactosamine (UDP–GalNAc) to threonine.[14,15] O-Linked glycoprotein modifications with xylose and fucose residues have also been observed in mammalian systems (not shown in Fig. 1).[16,17] In addition, proteins can be modified with a complex glycosyl phosphatidyl inositol (GPI) construct at the polypeptide carboxyl terminus; this derivatization confers new properties by anchoring the protein to membrane bilayers.[18] Substrates for GPI modification must terminate with one of six amino acids: Cys, Asp, Asn, Gly, Ala or Ser.[19] The α-carboxylic acid of these polypeptides is modified by an ethanolamine phosphate which is attached to a glycan with the sequence Man-α-1,2-Man-α-1,6-Man-α-1,4-$GlcNH_2$. The terminal glucosamine of this tetrasaccharide is attached to the C-6 of a *myo*-inositol ring which is linked to a diglyceride through a phosphate. The core structure, ethanolamine phosphate-Man-α-1,2-Man-α-1,6-Man-α-1,4-$GlcNH_2$-*myo*-inositol phosphate-diglyceride is conserved, however the length of the glyceride chains is variable. Further alterations of this sequence follow the initial modification and can include the addition of a second ethanolamine phosphate, additional monosaccharides, or the palmitoylation of the *myo*-inositol.[19]

Following protein glycosylation, the core oligosaccharide structures are subject to a sophisticated array of glycosyl transferase and hydrolase enzymes. For example, in the case of N-linked glycosylation, following transfer to an asparagine residue within the protein, the core tetradecasaccharide $GlcNAc_2$-Man_9-Glc_3 is immediately processed in a series of reactions which invariably cleave the three terminal glucose and four mannose residues. This processed glycoprotein is subsequently translocated to the Golgi where glycosyl transferases catalyze the elaboration of the glycoprotein through the transfer of additional monosaccharides such as fucose, galactose and sialic acid from the corresponding nucleotide diphosphates. These reactions act in concert to produce the diverse N-linked glycoconjugate structures characteristic of mature proteins.[20] The final oligosaccharide sequence often determines the cellular destination of the assembled glycoprotein. It is estimated that 100–200 glycosyl transferases are required to generate the diversity observed within glycoproteins.[5,21,22] Several of the enzymes involved in the assembly and cleavage of extended oligosaccharides have been isolated and characterized and significant progress has been made in developing a mechanistic understanding of these processes.[23,24] In contrast to carbohydrate trimming and elongation, details pertaining to the mechanism of the transfer of carbohydrate groups to the side chains of amino acids within proteins is less well understood. In particular, how do the enzymes involved in these processes mediate transfer to the target amino acid side chain in the presence of much more reactive moieties within the protein? Also, what factors govern the specificities of these transfers, as not all seemingly acceptable sequences undergo glycosylation? The mechanism of transfer to asparagine side chains is one of the most intriguing glycosylation reactions in this regard because of the seemingly inherent lack of reactivity of the carboxamide side chain. The intent of this review is to present the current understanding of oligosaccharyl transferase, the enzyme that catalyzes asparagine-linked glycosylation. Specifically, recent advances in the purification and characterization of the enzymatic complex, mechanistic and conformational elucidation of the reaction process and the conformational consequences of asparagine glycosylation will be discussed.

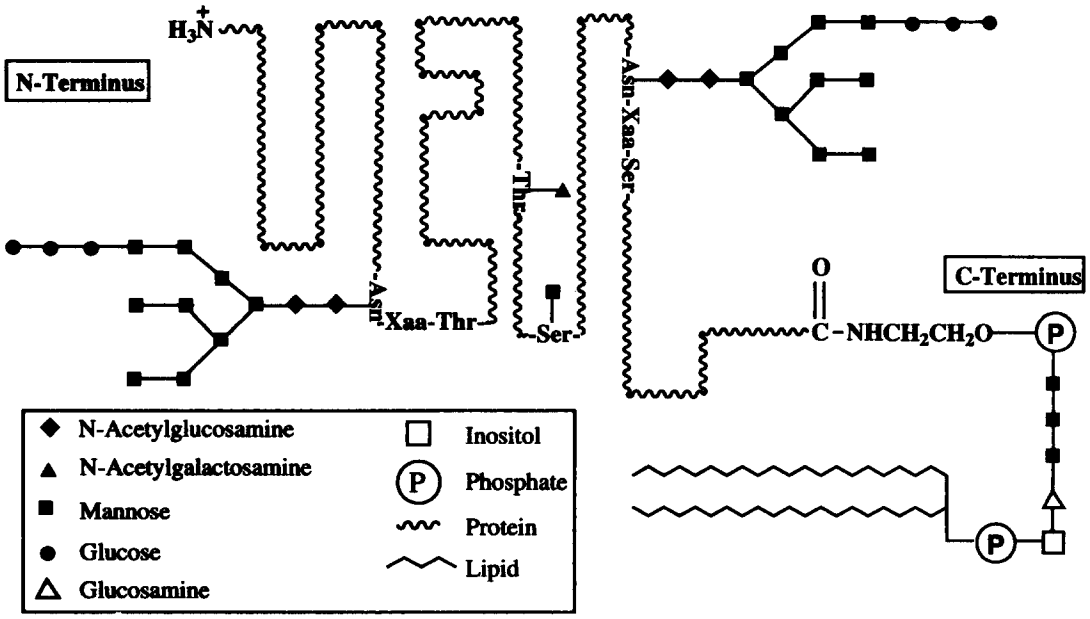


Figure 1. A schematic representation of the major types of carbohydrate–protein linkages. The core oligosaccharide structures (shown here) can be further modified through the addition of new monosaccharides or the cleavage of terminal saccharides.

Samsung Bioepis Exhibit 1091
Page 2

2. Asparagine-Linked Glycosylation

Oligosaccharyl transferase is a membrane associated, multimeric enzyme localized in the lumen of the endoplasmic reticulum. In the reaction catalyzed by OT, illustrated in Figure 2, a complex oligosaccharide is transferred from a lipid-linked pyrophosphate donor (1) to a nascent polypeptide chain as the peptide is translocated into the lumen of the endoplasmic reticulum. The peptide primary sequence requirements for glycosylation are minimal; the asparagine must reside within the consensus sequence Asn-Xaa-Thr/Ser (NXT/S) where Xaa can be any of the 20 natural amino acids except proline.[25–27] *In vivo*, threonine-containing sequences are almost three times more likely to be glycosylated than the corresponding serine-containing analogs.[28] However, the efficiency of NXT glycosylation *in vitro* exceeds that of NXS sequences by as much as 40-fold.[29] This first step in the N-linked glycosylation process appears to be conserved throughout eukaryotic evolution.[30]

3. Biochemical Characterization of Oligosaccharyl Transferase

Future research on the mechanism of action of OT will be greatly facilitated by the availability of a significant quantity of pure enzyme, however, purification efforts towards this end have been hindered by the inherent lability of enzyme activity during solubilization. Recently, significant advances have been made in achieving stable enzyme extracts, through the addition of phosphatidylcholine (PC),[31] sucrose or glycerol. In the presence of these additives, several research groups have succeeded in purifying OT to homogeneity; sequencing and analysis of several of the subunits have also been accomplished. Recently, OT has been purified from three mammalian sources (canine pancreas,[32] porcine liver microsomes[33] and human liver microsomes[34]), one avian source (hen oviduct microsomes)[35] and the yeast *Saccharomyces cerevisiae*[36–38] (see Table 1). Comparison of the protein subunits from each of these purifications evidences a high degree of conservation throughout evolution. In fact, a subunit of OT from the insect *Drosophila melananogaster* has recently been identified and sequenced using a PCR-mediated cloning strategy guided by the conserved regions of the canine and yeast enzymes.[39]

The first purification reported was that of the canine pancreas enzyme which was isolated as a trimeric complex of polypeptides with molecular weights of 66, 63 and 48 kDa.[32] The 48 kDa polypeptide has been designated as OST48 and is not homologous with any known proteins. Sequence comparisons and protein immunoblotting of the 66 and 63 kDa subunits demonstrated that they are identical to ribophorin I and ribophorin II, respectively (two previously identified integral membrane proteins found only in the rough endoplasmic reticulum).

The porcine liver OT has been purified as a tetrameric complex of polypeptides and is similar to the canine pancreatic enzyme.[33] Ribophorin I, ribophorin II and OST48 have all been identified, as well as an additional 40 kDa polypeptide not observed in the canine enzyme

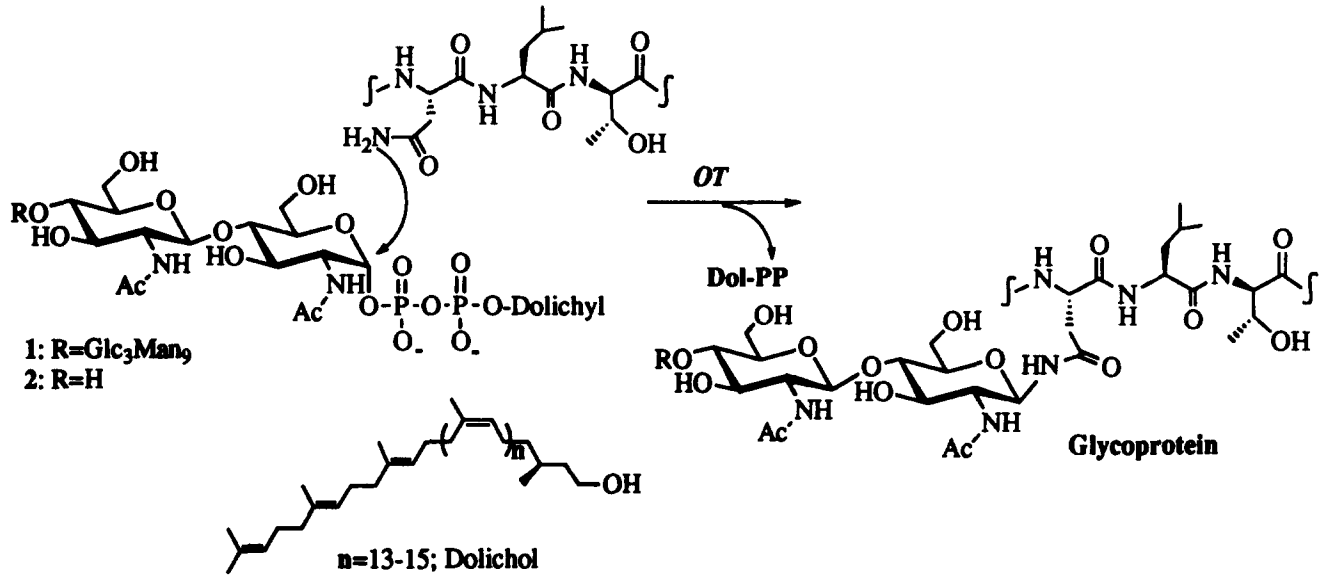


Figure 2. The glycosylation reaction catalyzed by oligosaccharyl transferase.

Table 1. Comparison of genetically characterized subunits of OT isolated from different sources

Mammalian (Canine[32,42]/Porcine[33]/Human[34])	Avian oviduct[35]	Yeast (*S. cerevisiae*)[43,44]
Ribophorin I (66/66/65 kDa)	Ribophorin I (65 kDa)	Nlt1p/Ost1p (64 kDa)
Ribophorin II (63/63/65 kDa)	Ribophorin II (65 kDa)	Swp1p (30 kDa)
OST48 (48/48/50 kDa)	OST48 (50 kDa)	Wbp1p (45 kDa)

Samsung Bioepis Exhibit 1091
Page 3

complex. Further purification to a dimeric complex of OST48 and ribophorin I yielded an unstable enzyme with partial activity. The remaining two subunits, ribophorin II and the 40 kDa polypeptide, are suggested to be involved in stabilizing the enzymatic complex.[33] Similar to the canine enzyme, the avian OT enzyme was purified as a trimeric complex of polypeptides with molecular weights of 65, 65 and 50 kDa.[35] The 50 kDa polypeptide was completely sequenced by overlaying peptide fragments obtained from cleavage by various different enzymatic and chemical methods. This sequence was found to be 92% identical to OST48. The two 65 kDa fragments were resistant to N-terminal sequencing, but cyanogen bromide cleavage followed by separation and sequencing of several fragments suggests that these two polypeptides correspond to ribophorin I and ribophorin II. The human OT enzyme was purified from liver microsomes and found to contain three subunits, identified as ribophorin I (66 kDa), ribophorin II (63 kDa) and a third subunit with a molecular weight of 50 kDa which was found to be 98% identical to the canine OST48.[34]

The multimeric enzyme complex in yeast has been isolated as both a tetrameric and a hexameric complex of polypeptides.[36–38] Two of the subunits have been identified as Wbp1p and Swp1p, with molecular weights of 45 and 30 kDa, respectively. The remaining polypeptides have molecular weights of 64 and 34 kDa in the tetrameric complex[30,36] and 64, 34, 16 and 9 kDa in the hexameric complex.[37] Independent depletion studies have indicated that both Wbp1p and Swp1p are absolutely required for *in vivo* and *in vitro* OT activity.[40,41] Additionally, the two subunits can be chemically crosslinked suggesting that the polypeptides are members of the same multisubunit complex.[41] Comparison of the yeast and mammalian enzyme complexes reveals a remarkable degree of similarity: Wbp1p exhibits 25% sequence homology to OST48;[42] the 64 kDa polypeptide, designated Nlt1p or Ost1p, is homologous to ribophorin I;[30,43,44] Swp1p is similar to the carboxyl-terminal half of ribophorin II;[37] a peptide which would be analogous to the amino-terminal half of ribophorin II has not yet been identified in yeast.

Three of the subunits of the yeast enzyme complex, Wbp1p,[45] Swp1p,[41] and Ost1p[43]/Nlt1p,[44] have been sequenced and characterized. The results of these studies are summarized in Figure 3. Each of the subunits contains at least one C-terminal hydrophobic domain. Additionally, each polypeptide includes an amino terminus signal sequence (17–20 amino acids) which is cleaved prior to complete maturation of the protein. Wbp1p and Nlt1p are both multiply glycosylated with N-linked high mannose oligosaccharides, indicating that mature OT is a self-processing enzyme.[37] In fact, concanavalin A (a high mannose binding lectin) was successfully used to partially purify OT in two of the procedures described above.[36,37]

The mammalian OST48 subunit and its counterpart, the yeast Wbp1p have been further characterized through two separate sets of experiments. As mentioned above, the porcine enzyme was purified to a partially active, unstable dimeric complex of OST48 and ribophorin I.[33] Treatment of this complex with trypsin demonstrated that ribophorin I was more susceptible to proteolysis than OST48; partial activity was retained during proteolysis suggesting that OST48 is the catalytically competent subunit. However, this experiment does not rule out the participation of a proteolytic fragment of ribophorin I. In independent chemical modification studies, Wbp1p, the analogous subunit of the yeast enzyme, was modified by *N*-biotinoyl(aminoethane)thiolsulfonate (**3**, BMTS), a biotinylated analog of methyl methanethiolsulfonate (**4**,

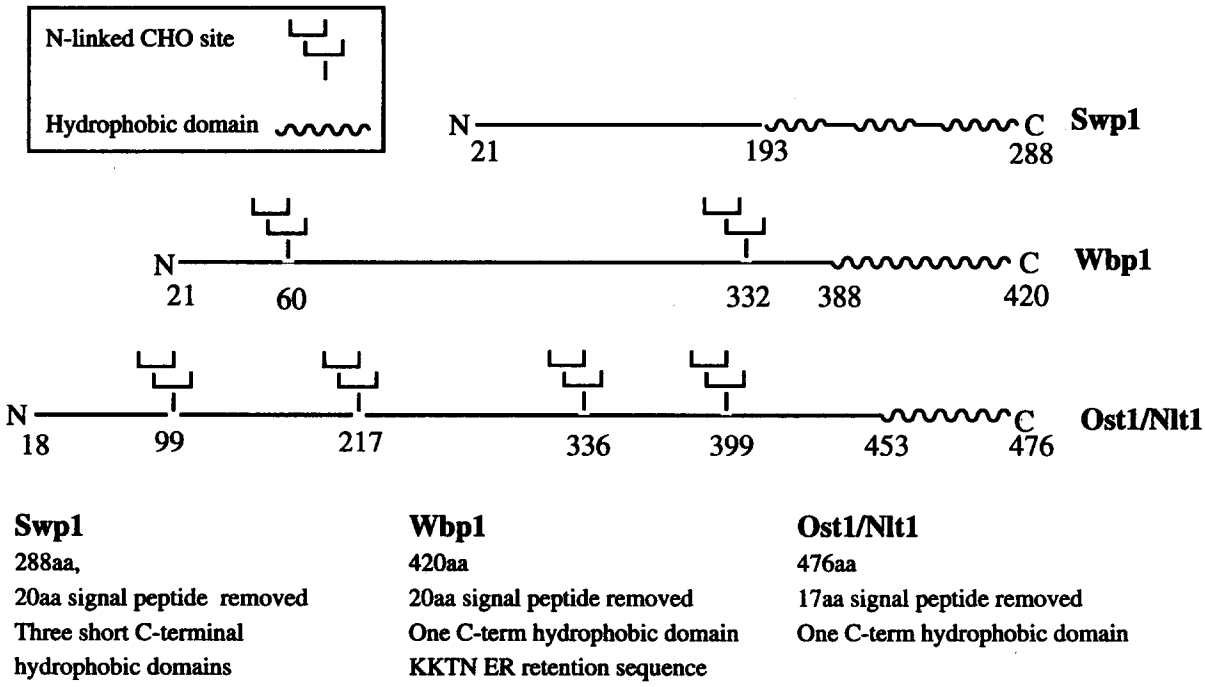
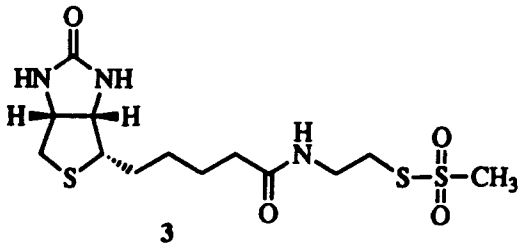


Figure 3. The current understanding of the subunit composition of oligosaccharyl transferase.

Samsung Bioepis Exhibit 1091
Page 4

MMTS).[36] OT was found to be inactivated by both MMTS and BMTS. Following BMTS modification and separation of the subunits of OT by SDS-PAGE, visualization of the biotin moiety by a conjugated avidin system revealed that Wbp1p was the only subunit modified by BMTS. Substrate protection experiments indicated that the rate of inhibition was hindered by preincubation of the enzyme with the dolichol-PP-GlcNAc$_2$ substrate (2), suggesting that this subunit contains the oligosaccharide binding site and possibly the catalytic site for the enzyme complex.



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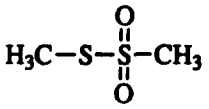

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4. Substrates for N-Linked Glycosylation

4.1 Lipid-linked oligosaccharide donor

The normal physiological glycosylation process results in the transfer of a triantennary branched oligosaccharide made up from two *N*-acetyl glucosamine, nine mannose and three glucose residues (1, Dol-PP-GlcNAc$_2$-Man$_9$-Glc$_3$, Fig. 2).[46,47] This tetradecasaccharide derivative is biosynthesized through a series of stepwise transfers to dolichol phosphate before being transferred *en bloc* to the peptide acceptor.[48,49] The first step in the biosynthesis of the lipid-linked donor involves transfer of a single *N*-acetyl glucosamine from UDP–GlcNAc to dolichol phosphate and the formation of a pyrophosphate bond with concomitant release of UMP. The second GlcNAc is subsequently transferred to the newly formed dolichol-PP-GlcNAc in a reaction that results in the formation of a β-1,4-glycosidic linkage. The remaining 12 sugars are subsequently added to this lipid-linked disaccharide. The first five mannose residues are directly transferred from GDP–Man, while the last four mannose residues, as well as the terminal glucose residues (from UDP–Glc) are synthesized via the intermediacy of a dolichol phosphate derivative before being added to the assembling glycolipid. The oligosaccharide of the final glycolipid is located on the lumenal side of the ER.[50,51]

Although the substrate recognized by OT *in vivo* is a complex tetradecasaccharide, the enzyme catalyzed reaction *in vitro* proceeds with similar efficiencies utilizing simpler truncated substrates such as the di-*N*-acetylglucosamine derivative 2 (Dol-PP-GlcNAc$_2$, Fig. 2) and Dol-PP-GlcNAc$_2$-Man$_1$. However, the lipid-linked monosaccharide, Dol-PP-GlcNAc, is not a substrate for the solubilized enzyme.[49] The ability of OT to readily utilize a simple lipid-linked disaccharide as the carbohydrate donor in the glycosylation of asparagine residues has greatly facilitated the study of this enzyme. These truncated substrates are accessible through synthetic and enzymatic methods and radiolabeled monosaccharides can be incorporated into the desired compounds for enzymatic assays. The chitobiosyl analog 2 has been chemically synthesized by two different methods.[52,53] These synthetic methods are hindered by availability of reactants and difficulty in purification. Compound 2 has also been prepared enzymatically by incubating liver microsomes with radiolabeled UDP–GlcNAc in the presence of divalent magnesium. The reactions catalyzed by the microsomal enzymes are as follows.

$$\text{DolP} + \text{UDP-GlcNAc} \xrightarrow{\text{Enzyme I}} \text{Dol-PP-GlcNAc} + \text{UMP}$$

$$\text{Dol-PP-GlcNAc} + \text{UDP-GlcNAc} \xrightarrow{\text{Enzyme II}} \text{Dol-PP-(GlcNAc)}_2 + \text{UDP} \quad (2)$$

The biosynthesis of 2 from dolichol phosphate and UDP–GlcNAc appears to be limited due to low conversions to the desired lipid-linked mono- and disaccharides. This technical problem has recently been overcome by applying a combination of chemical and enzymatic methods. Efficient preparations of lipid-linked substrates for assaying N-linked glycosylation are generated by treating synthetic Dol-PP-GlcNAc with UDP–GlcNAc, in the presence of divalent magnesium and liver microsomes.[54] This process results in conversions in excess of 60% and allows for the selective incorporation of radiolabeled GlcNAc (^{3}H at C-6) at the terminal saccharide position; the specific activity of the substrate can therefore be varied as needed.

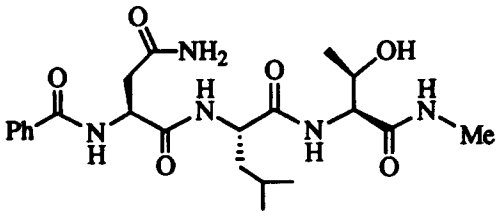
4.2 Peptide acceptor

The other substrate for N-linked glycosylation is a nascent polypeptide chain containing an asparagine residue within an NXT/S consensus sequence. As previously stated, OT does not accept peptides which contain proline as the middle residue in the NXT/S triad. The exclusion of NPT/S sequences suggests that local secondary structure plays an important role in determining the outcome of glycosylation. This theory is further underscored by the fact that 10–30% of all NXT/S sequences remain unglycosylated in mature proteins, even though they meet the primary sequence requirements.[28,55] Proteins that are destined for glycosylation are synthesized by membrane-associated ribosomes with an initial signal peptide (approximately 16 residues) which guides the newly synthesized protein through the intracellular membrane bilayer into the lumen of the ER. The signal peptide is subsequently cleaved by a signal peptidase. The NXT/S sequence must clear the lumenal side of the membrane bilayer by 12–14 residues before glycosylation can occur,

Samsung Bioepis Exhibit 1091
Page 5

however, the nascent polypeptide remains bound to the membrane of the ER and is likely to be only locally folded during OT mediated catalysis.[56] In fact, a direct correlation between lack of tertiary structure and glycosylation acceptor ability has been observed by subjecting unglycosylated NXT/S sequences in native proteins to varying levels of reducing and denaturing conditions and then re-examining these unfolded proteins for substrate behavior with OT. In many cases, the proteins did not exhibit any glycosyl acceptor properties prior to unfolding; however, three out of seven of the proteins examined were efficiently glycosylated following sulfitolysis.[57]

In the cellular process, OT recognizes an extended peptide sequence as it crosses the membrane bilayer, however, the tripeptide recognition sequence is sufficient for *in vitro* glycosylation, provided the two termini are capped by protecting groups which mimic the amide backbone of an extended peptide. With synthetic tripeptides, OT will glycosylate peptides capped with benzoyl (Bz), octanoyl, butanoyl, acetyl (Ac), carbobenzyloxy (Cbz) and *tert*-butoxycarbonyl (Boc) groups at the amino terminus, although the carbamate modifications of the Cbz and Boc groups yield very poor substrates.[58–61] The carboxy terminus is most effectively protected as a primary or secondary amide, although methyl esters are tolerated. The tripeptide Bz-Asn-Leu-Thr-NHMe (**5**, Bz-NLT-NHMe), where the amino terminus has been protected with a benzoyl group and the carboxy terminus as a methyl amide, is an effective synthetic tripeptide for *in vitro* investigations.[61]



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The tripeptide consensus sequence has been thoroughly investigated by selective replacement of each amino acid with key natural and synthetic amino acids and re-examination of the substrate behavior of the new tripeptide analog. These studies have revealed that the first position in the sequence almost invariably requires an asparagine residue. Tripeptides containing glutamine or N^{δ}–methylasparagine are not tolerated by the enzyme nor do they inhibit the glycosylation of natural sequences.[61] Tripeptides containing β-fluoroasparagine are very poorly glycosylated, at a rate of less than 2% that of Bz-Asn-Leu-Thr-NHMe.[62] However, the enzyme has been shown to tolerate a tripeptide which contains a thioasparagine (Asn(γS)) residue in the first position. This compound exhibits a similar K_{Mapp}, but has a greatly reduced V_{max} when compared to Bz-Asn-Leu-Thr-NHMe.[63] The second position in the consensus tripeptide may include most of the 20 natural amino acids except proline; in addition, incorporation of aspartic acid as the center residue depresses glycosyl acceptor efficiency. The unnatural amino acids α-aminoisobutryic acid and D-alanine in the central position are not recognized.[59] The third position, a hydroxy amino acid in the natural substrate sequences, is also invariant. Tripeptides containing Val,[29] Thr(β-OMe),[29,64] β-hydroxynorvaline[65] and *allo*-Thr[66] in the C-terminal position are not recognized by OT. Peptides which incorporate cysteine in this position show a low, but measurable, level of glycosyl acceptor activity.

5. Conformational Requirements

Oligosaccharyl transferase exhibits a simple requirement for peptidyl substrates containing the NXT/S consensus sequence, yet it catalyzes an unusual and specific reaction wherein the nucleophilicity of the asparagine side chain is greatly enhanced. In addition to the asparagine residue, the absolute requirement for an unmodified hydroxyl amino acid implies a direct role for the hydroxyl group in catalysis. Beyond these straightforward tripeptide requirements, additional features are important since many seemingly acceptable NXT/S sequences still remain unglycosylated following translation and translocation into the lumen of the ER.[28,55] Understanding the potential role of substrate conformation in contributing to the specificity of the reaction is a prerequisite for developing a satisfactory mechanistic model. At the time of glycosylation, the peptide substrate is relatively free of tertiary structure, although local secondary structural motifs are still available as recognition elements. Since tripeptides are accepted as substrates, the length of the consensus triad primarily limits the types of available hydrogen-bonded motifs to simple turns, since other structural features such as α-helices or β-sheets tend to require longer peptides for complete formation. The tripeptide substrates would be compatible with two types of turns: the β-turn and the Asx-turn. These two turns are illustrated in Figure 4 for the peptide Ac-Asn-Xaa-Thr-NHMe. The β-turn forms a complete chain reversal, and is characterized by a hydrogen bond between the threonine amide and the carbonyl immediately preceding the asparagine. In the case of Ac-Asn-Xaa-Thr-NHMe, the hydrogen-bonding carbonyl is provided by the acetyl group. The Asx-turn involves a hydrogen-bonding array similar to that of a β-turn, however, in this case, the hydrogen bond acceptor is the carbonyl oxygen of the asparagine side chain. The polypeptide backbone has a more extended conformation and does not experience a complete chain reversal. Approximately 18% of all asparagine and aspartic acid side chains appear to be involved in Asx-turns in proteins.[67] In fact, a statistical survey of globular proteins by Baker *et al.*[68] indicates that 55% of all hydrogen bonds to the carbonyl side chain of asparagine residues are provided by the backbone NH of the $(i + 2)$ residue. It is noteworthy that the homologous residue glutamine is never glycosylated and this apparent contradiction in reactivity may be explained by the distinct conformational preferences of this residue. Specifically, the carboxamide side chain of glutamine is seldom involved in short range hydrogen-bonding interactions. The possibility that the Asx-turn or the β-turn is the unique secondary motif preferentially recognized by OT has been considered as an explanation for both the

Samsung Bioepis Exhibit 1091
Page 6

specificity of the enzyme and the enhanced nucleophilicity of the peptidyl substrate, particularly in model mechanisms which rely on secondary structure to position the hydroxyl moiety within direct proximity to the asparagine side chain.[25,63,69]

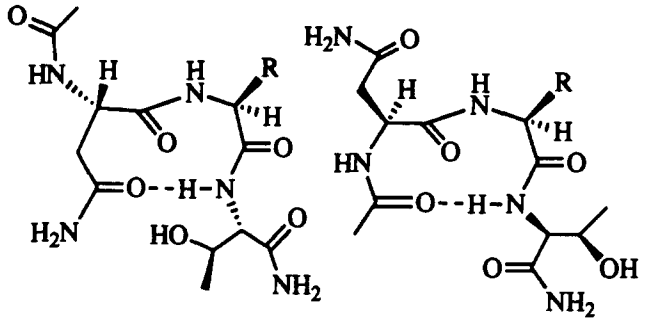
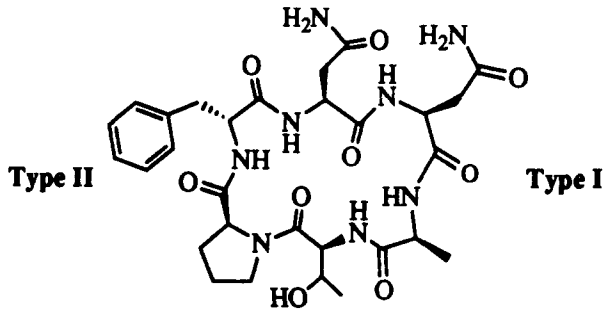


Figure 4. A comparison between an Asx-turn and a β-turn for the tripeptide Ac-Asn-Xaa-Thr-NH_2.

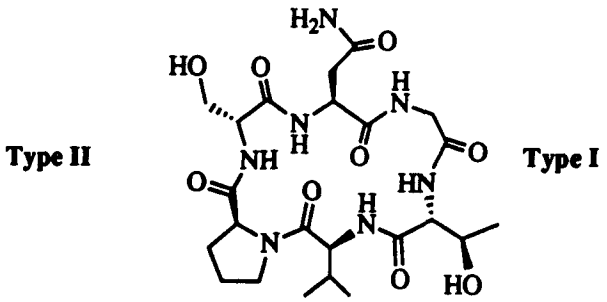
The ability of OT to recognize and glycosylate short peptidyl substrates has enabled researchers to investigate the structural requirements for glycosylation by designing molecules which contain all of the recognition elements of the consensus sequence, but which are constrained or restricted to specific conformations. Kinetic analyses of these compounds have provided a detailed understanding of the role of conformation in asparagine-linked glycosylation.

Initial studies correlating substrate–acceptor properties and solution state conformation suggested that the Asx-turn might be the key recognition motif.[59] To rigorously distinguish between the Asx-turn and the β-turn, a study was carried out which placed the reactive tripeptide sequence with asparagine in either the (i) or (i + 1) position of a constrained type I β-turn that had been built into the architecture of a cyclic hexapeptide (**6a** and **6b**).[58] The type I β-turn was fixed by the incorporation of a prolyl-D-amino acid dipeptide at the non-reactive end of the cyclic compound. This dipeptide sequence imposes a constrained type II β-turn in the cyclic peptide and therefore limits the conformations accessible to the stucture.[70] One and two dimensional NMR studies were utilized to verify the turn conformations of compounds **6a** and **6b**. These two compounds were assayed for substrate activity with OT and compared to two standard peptides, Ac-Asn-Leu-Thr-OMe and Ac-Asn-Leu-Thr-NHMe. The results of these studies are summarized in Table 2. With the reactive asparagine conformationally constrained into a β-turn (as in compounds **6a** and **6b**), the resultant peptides did not exhibit any detectable substrate behavior with OT.

In complementary studies, a constrained Asx-turn was incorporated into a cyclic NXT sequence, c[Asn-Add]-Thr-NHMe (**7**) through a side chain to main chain lactam cyclization. An unnatural amino acid, (S)-2-amino-decanedioic acid (Add), was utilized in the central position to provide functionality for cyclization with the amino terminus.[71] This cyclization was selected because modeling studies indicated that it would constrain the critical Asn ψ and Xaa ϕ dihedral angles to generate an Asx-turn. The presence of an Asx-turn was confirmed through NMR experiments on an aqueous solution of the peptide (43% methanol). The peptide was analyzed for substrate activity with OT and compared to the linear analog, N^{α}-Butanoyl-Asn-Leu-Thr-NHMe (**8**). Introduction of a cyclic constraint improved the K_m 10-fold, from 800 μM for **8** to 78 μM for **7**, indicating that the preorganization provided by the Asx-turn enhanced enzyme/substrate affinity. The relative maximal velocities of the two compounds remained similar. The enhancement of enzyme affinity for the constrained peptide, **7**, with a well-defined Asx-turn conformation firmly demonstrates the importance of the Asx-turn over the β-turn, as the recognition motif for OT.



6a



6b

Table 2. Kinetic analysis of conformationally constrained peptides with porcine liver OT[58]

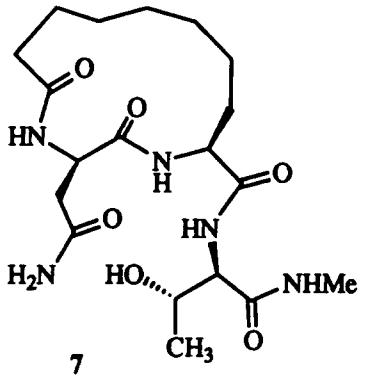
Peptide	Apparent K_m(mM)	Relative V(%)[a]
Ac-Asn-Leu-Thr-OMe	6.7	100
Ac-Asn-Leu-Thr-NHMe	0.3	150
c(Pro-D-Ser-Asn-Gly-Thr-Val) (**6a**)	>20	
c(Pro-D-Phe-Asn-Asn-Ala-Thr) (**6b**)	>20	

[a]Ac-Asn-Leu-Thr-OMe as standard.

In addition, circular dichroism (CD) studies were carried out on **7**, **8** and N^{α}-Butanoyl-Gln-Leu-Thr-NHMe (**9**). The spectrum obtained for **9**, which is not a substrate for OT, resembled that of a random coil (Fig. 5) with a strong negative ellipticity at 198 nM. However, the CD spectrum for **7** was quite distinct, with a greatly diminished negative ellipticity at 198 nm and a strong signal at 218 nm, providing complementary spectroscopic evidence that the cyclic constraint promoted ordered secondary structure. These results were independent of the solution com-

Samsung Bioepis Exhibit 1091
Page 7

position which was varied from 2 to 43% methanol in water. While the CD spectrum for **8** was primarily random coil, a small signal was present at 218 nm indicating the presence of some secondary structure.[71]



8: N^{α}-Butanoyl-Asn-Leu-Thr-NHMe
9: N^{α}-Butanoyl-Gln-Leu-Thr-NHMe

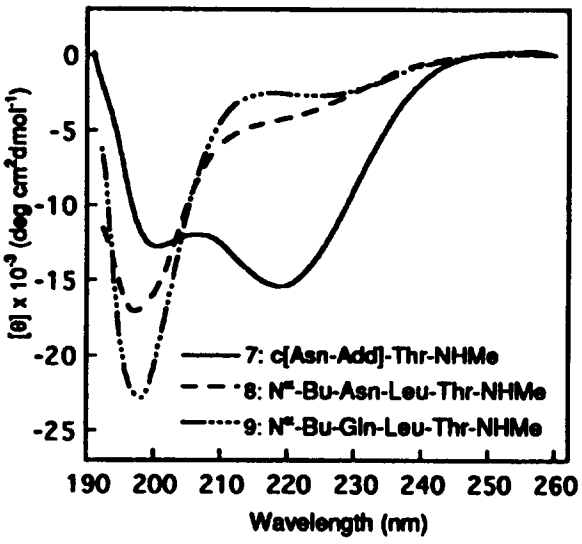


Figure 5. CD spectra of constrained and linear tripeptide substrates for oligosaccharyl transferase.[71] Samples were prepared by dissolving each tripeptide in a 40% methanol/water solution at pH 4.5. In each case, the final peptide concentration was 500 μM.

Previous studies by Bause[72] had examined the effects of disulfide constraints on glycosylation acceptor properties. In all cases where the presence of the disulfide enhanced a β-turn structure, glycosyl acceptor properties diminished. In light of the proposal that an Asx-turn is the recognition motif for N-linked glycosylation, re-examination of the Bause results support the hypothesis that acceptor ability of an NXT sequence is related to peptides which can access an Asx-turn motif. With a complete understanding of the necessary secondary structural features required for N-linked glycosylation, mechanistic investigations can be designed to consider the roles of the hydroxy amino acid and the amide backbone in enhancing the nucleophilicity of the asparagine side chain.

6. Mechanistic Considerations

To complete N-linked glycosylation, cooperation between OT and the hydrogen-bonding array provided by the peptidyl substrate results in the activation of a normally non-nucleophilic amino acid. Currently, the generation of this unusual reactivity is the main focus of mechanistic investigations. Evidence in support of, or against different mechanistic proposals is most readily obtained by examining the kinetic properties of substrate analogs. By selectively altering different features of a natural substrate to generate a nonnatural substrate or an inhibitor, information about specific features of the active site, or of the reaction intermediates can be surmised.

Initially, Marshall[25] proposed that the acidity of one of the asparagine amide protons is increased by the presence of a hydrogen bond between the hydroxyl moiety of the threonine or serine and the carbonyl of the carboxamide side chain (Fig. 6a). This interaction would increase the acidity of the carboxamide protons and thereby facilitate ionization to afford a nucleophilic anionic nitrogen species that could subsequently engage in the glycosylation event. Bause[29] proposed a different hydrogen-bonding array wherein one of the carboxamide protons of the asparagine side chain serves as a proton donor to the hydroxyl group of the threonine or serine, thus increasing the nucleophilicity of the carboxamide nitrogen (Fig. 6b). Both of these mechanisms rely on the ability of the tripeptide substrate to position the hydroxyl moiety within hydrogen-bonding distance of the asparagine side chain.

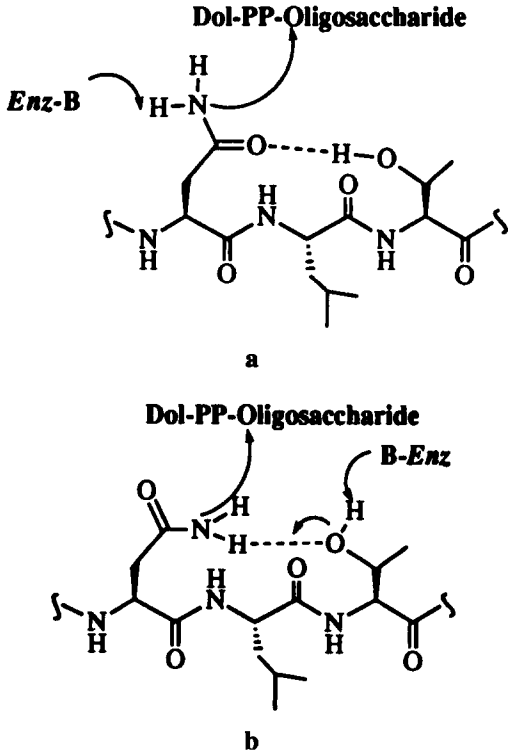


Figure 6. Schematic representations of two different activation mechanisms for oligosaccharyl transferase. Figure 6a, proposed by Marshall.[25] Figure 6b proposed by Bause.[29]

Two different peptides have been examined which incorporate an aspartic acid in place of the required asparagine residue within the consensus triad. If, during the reaction course of glycosylation, a negative charge is developed on the asparagine nitrogen, then the aspartate replacement would potentially mimic the charge distribution of this intermediate and therefore afford a competitive inhibitor. The peptides, Tyr-Asp-Leu-Thr-Ser-Val[29] and Bz-Asp-Leu-Thr-NHMe (**10**, Table 3), do not inhibit OT, even at high concentrations (> 5 mM) although each would be expected to maintain the hydrogen-bonding array of an Asx-turn;[63] this result suggests that a negative charge is not developed at this center during the reaction process.

An additional mechanism for asparagine linked glycosylation has been proposed which incorporates aspects of the favored mechanism for some of the glutamine amidotransferase family of enzymes.[73] This proposal[60] involves the release of an active site bound 'NH_3' from the asparagine side chain in conjunction with the formation of a cyclic isoasparagine intermediate. The activated ammonia reacts with the lipid-linked oligosaccharide donor to form chitobiosyl amine which re-opens the isoasparagine to complete the glycosylation event. However, synthetic preparations of the isoasparagine (isoAsn) containing tripeptide Bz-isoAsn-Leu-Thr-NH_2 revealed that this compound is neither a substrate for nor an inhibitor of OT. In addition, this mechanistic proposal does not consider the absolute requirement for a hydroxyl amino acid.

The conformational studies identifying the Asx-turn as the likely recognition motif for asparagine linked glycosylation have prompted the development of a new mechanistic proposal for OT-mediated catalysis.[63] In this proposal, the unique hydrogen-bonding array provided by the Asx-turn is suggested to facilitate protonation of the carbonyl of the asparagine side chain. Enzyme-mediated deprotonation at the nitrogen subsequently induces the tautomerization of the carboxamide to the imidol. This tautomerization would yield the neutral nucleophilic species which could then react with the electrophilic lipid-linked oligosaccharide (Fig. 7). This mechanistic proposal incorporates both the issues of specificity and reactivity as well as the absolute requirement for a hydroxyl amino acid by integrating substrate structural requirements with participation of enzyme active site residues. Thus, the likelihood of an NXT/S sequence to undergo glycosylation would be governed by the ability of each potential substrate to adopt an Asx-turn conformation within the active site of the enzyme.

Four tripeptide analogs of **3** were designed to probe different aspects of the OT peptide binding site during catalysis; a summary of the kinetic analyses of these compounds is listed in Table 3.[63] Bz-Asp-Leu-Thr-NHMe (**10**), as discussed previously, exhibited no binding to OT. Replacement of the asparagine carboxamide with a methyl ester, in Bz-Asp (Oγ̈Me)-Leu-Thr-NHMe (**11**), removes the ionizable group from the side chain and affords a tripeptide which is not recognized by OT. Incorporation of γ-aminobutyrine (Amb) in place of asparagine yields Bz-Amb-Leu-Thr-NHMe (**12**), a peptide with a significantly lower pK_a at the Amb side chain. Tripeptide **12** is a competitive inhibitor of OT with a K_i that is similar to the K_m of **5**; the increased acidity of the side chain in **12** apparently facilitates binding to the enzyme and compensates for the peptide not having the appropriate functionality to adopt an Asx-turn. Finally, the tripeptide Bz-Asn (γS)-Leu-Thr-NHMe (**13**) is a substrate for OT. This heteroatom replacement ($CSNH_2$) increases the acidity of the hydrogen-bond donor site and decreases the basicity at the hydrogen-bond acceptor site relative to the corres-

Table 3. Kinetic analysis of tripeptides with porcine liver OT[63]

Peptide	Apparent K_m (mM)	Relative V(%)[a]	K_i (mM)
Bz-Asn-Leu-Thr-NHMe (**5**)	0.24	100	
Bz-Asp-Leu-Thr-NHMe (**10**)			>10[b]
Bz-Asp(Oγ̈Me)-Leu-Thr-NHMe (**11**)			>10[b]
Bz-Amb-Leu-Thr-NHMe (**12**)			1.0
Bz-Asn(γS)-Leu-Thr-NHMe (**13**)	0.26	8.4	

[a]Peptide **2** as standard; [b]no inhibition observed at concentrations below 5 mM.

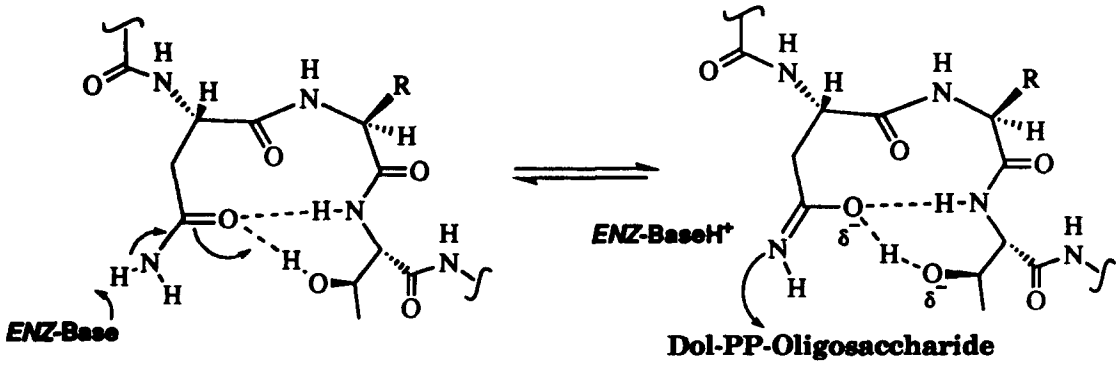
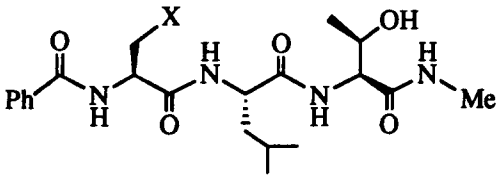


Figure 7. Mechanism of activation for oligosaccharyl transferase as proposed by Imperiali.[63]

ponding oxoamide ($CONH_2$).[74,75] Additionally, the sulfur substitution introduces some steric perturbations into the system by increasing the double bond length (C=S > C=O) and atomic radius (S > O) by approximately 25% relative to the corresponding carboxamide.[74] The binding of **13** to OT is scarcely affected by the thiocarbonyl replacement which is consistent with the ability of this compound to adopt an Asx-turn-like conformation. However, the relative maximal velocity of **13** in a standard OT assay is reduced to 8.4% of that observed for **2**.



10: X=CO_2H

11: X=CO_2Me

12: X=$CH_2NH_3^+$

13: X=$CSNH_2$

Together, the results obtained from this substrate analog study suggest that in order for recognition by OT to occur, there is a prerequisite for side chain functionality with ionization properties similar to those of the native substrate. Tripeptide **12** is readily deprotonated and is a competive inhibitor, while **13** is a substrate for the enzyme. For **12**, the lower pK_a of the nitrogen appears to have allowed recognition by OT, despite the loss of the Asx-turn motif. For peptide **13**, the K_m remains similar to that of the native substrate, however the change in electronic properties have resulted in a lower glycosylation rate. These results support mechanistic proposals which do not involve the generation of a nitrogen anion and are consistent with the mechanism proposed by Imperiali and coworkers[63] which suggests that an intricate interplay between protonation and deprotonation is critical for the enhanced reactivity of the asparagine side chain. The microenvironment afforded by the hydrogen-bonding array of the Asx-turn provides the ideal arrangement to facilitate tautomerization. The presence of an active site base is a central element of this proposal as deprotonation of the imidol tautomer would result in the formation of a competent nucleophilic species.

An additional comparative analysis was also carried out using the natural substrate Bz-Asn-Leu-Thr-NHMe (**2**) and the modified substrate Bz-Asn(γS)-Leu-Thr-NHMe (**13**) with OT that had been separately reconstituted with each of four metal cations: Mn^{2+}, Fe^{2+}, Mg^{2+} and Ca^{2+} (Table 4).[76] OT is dependent on divalent metals for activity and functions most efficiently with Mn^{2+}.[77] However, following exhaustive depletion of all divalent metal cations which is accompanied by a complete loss in transferase function, total or partial enzyme activity can be regenerated by the addition of several different divalent metals, including the four mentioned above. Kinetic analyses of **2** and **13** revealed that **2** is a substrate for OT in the presence of each of the four metal cation cofactors, whereas **13** only exhibits glycosyl acceptor properties when the enzyme is reconstituted with the thiophilic metal cations Fe^{2+} or Mn^{2+}.

Additionally, steric effects due to the differences between the ionic radii of the metals and of the sulfur and oxygen atoms in the asparagine side chain were observed. The fact that the size and thiophilicity of each metal cation affected the kinetic behavior of each of the two peptides differently suggests that the metal cation is involved in interactions with the peptidyl substrate during catalysis.

As a substrate for OT, the thioamide **13** has the ability to undergo glycosylation at either the nitrogen or the sulfur of the thioasparagine side chain. Although glycosylation of the nitrogen would be analogous to the reaction of the natural oxoamide substrate, the non-enzymatic reactivity of thioamides suggests that in this case alkylation at sulfur rather than the nitrogen might be favored.[78] A detailed analysis of the glycopeptide product of **13** however confirmed that a minimum of 70% of the glycopeptide product resulted from alkylation at nitrogen. No indication of products resulting from sulfur alkylation was observed.[76] Thus, the structural features imposed by the Asx-turn and the peptide binding site of OT appear to prohibit sulfur glycosylation in spite of the inherent reactivity of the functional group.

The studies with the thioasparagine-containing peptide have implications for the native glycosylation reaction suggesting that the enzyme active site and the Asx-turn act together to provide a unique environment at the glycosylation site that contributes to the regioselectivity of amide alkylation. This precise regiochemical control is essential to prevent glycosylation at oxygen since the resultant O-alkylated imidates would be too labile to be viable in a physiological context. Additionally, this same regiochemical control prohibits incorrect glycosylation of other

Table 4. Kinetic parameters for **5** and **13** in the presence of various divalent metal cations[76]

	Bz-Asn-Leu-Thr-NHMe (**5**)		Bz-Asn(γS)-Leu-Thr-NHMe (**13**)	
Metal ion (10 mM)	Apparent K_m (μM)	V_{max} (dpm min^{-1})	Apparent K_m (μM)	V_{max} (dpm min^{-1})
Mn^{2+}	380	4550	380	326
Fe^{2+}	650	2120	680	703
Ca^{2+}	1100	4800	N/A[a]	No Rate
Mg^{2+}	2500	2930	>5000	<100

[a]Observed rates were not above background.

potentially reactive amino acids. For example, the sequence -Gln-Xaa-Thr- is found in nature, but is never glycosylated by OT. This sequence is not predisposed to form a structure that is analogous to an Asx-turn since the corresponding hydrogen-bonded network is less stable. Without the ability to adopt a 'Glx' turn, the mechanistic machinery used by OT to activate the asparagine side chain cannot similarly activate the glutamine. The unique reaction of asparagine in N-linked glycosylation is thereby governed by both the enzyme active site and the propensity of substrates to specifically adopt an Asx turn.

7. Conformational Consequences of Co-translational Protein Glycosylation

Thus far, this report has focused on the conformation of the peptidyl acceptor prior to glycosylation. However, since glycosylation is a co-translational event, the impact of the modification on the final folded protein structure and stability are of current interest. Comparisons between glycosylated and non-glycosylated peptides and proteins can offer information on the consequences of the modification on the conformation of the peptide and the oligosaccharide. In many cases, the effects of N-linked glycosylation on the microenvironment of the asparagine residue has been directly observed. Several different techniques are available for analysis of glycopeptide structure including NMR[79–81] and CD spectroscopies[82–84], molecular modeling predictions[84,85] and fluorescence energy transfer (FET) experiments.[7] These types of analyses have been performed on proteins and smaller peptide fragments.

Nuclear Overhauser effects (NOE), amide temperature coefficient and deuterium isotope exchange kinetics experiments have all been used to investigate the structural effects of protein glycosylation. The structure of a glycosylated 22 residue peptide fragment, isolated from human serum immunoglobulin M and containing one NVS glycosylation site, was investigated using selected NOE and amide temperature coefficient studies.[79] The isolated polypeptide contained three different glycoforms at the asparagine side chain which varied in mannose content from six to nine residues. Comparisons of the NOE data from the isolated glycopeptide and the analogous, synthetic, non-glycosylated peptide revealed that the presence of the oligosaccharide decreased the conformational mobility of both the backbone and the side chains of the amino acids immediately surrounding the glycosylation site. In addition, the amides in this same region were significantly more solvent shielded in the glycopeptide. Analysis of the coupling constant between GlcNAc1–C1H and Asn9–NδH revealed that the protein–glycan linkage is rigid and planar. The presence of the three different glycoforms did not affect the resolution of the peptide peaks, suggesting that protein conformation is not directly affected by the terminal saccharide residues.[79] Additionally, hydrogen isotope exchange experiments were used to compare the structures of RNase A and its glycosylated counterpart RNase B.[80] Although the presence of the oligosaccharide did not dramatically affect the global protein conformation, glycosylation did enhance the stability of the molecule. This result was evidenced by the decreased deuterium/proton exchange rates observed for several of the amides, even those buried within the protein and away from the oligosaccharide in RNAse B.[80]

Circular dichroism (CD) spectroscopy provides less direct structural information than NMR experiments, however it requires significantly less sample and can still be used to observe dramatic conformational differences.[82,83] Analysis of eight pairs of model peptides and glycopeptides by CD has revealed that in hydrophobic solvent systems like trifluoroethanol or acetonitrile, glycosylation results in a decrease in type I β-turn character and a concomitant increase in type II β-turn character.[84] This trend is reversed when the CD spectra are acquired in aqueous environments. One possible explanation for this observation is that in the more hydrophobic environments the oligosaccharide interacts with the peptide backbone and side chains through a hydrogen-bonding array; when the sample is in an aqueous environment, the medium provides competition for these hydrogen bonds.

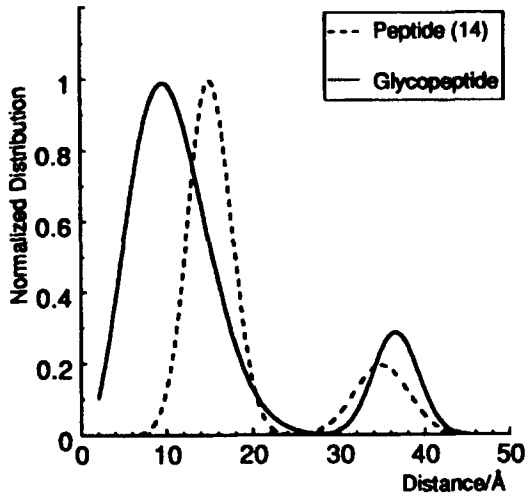


Figure 8. Dynamic distribution plots of **14** and corresponding glycopeptide in aqueous solution.[7]

Fluorescence energy transfer experiments (FET) can also be used to investigate the conformational consequences of glycosylation. FET is only suitable for peptides which can be modified to incorporate a fluorescent donor and acceptor. Generally, the indole side chain of the naturally occurring amino acid tryptophan is employed as the donor fluorophore. Similar to CD, FET requires minute quantities of sample (nmol) and is useful for observing gross structural changes in peptide conformations. However, unlike CD, FET can be used to determine the distances between acceptor and donor moieties. Additionally, FET measurements can be made on the same time scale as that for conformational fluctuations within the molecule, so the data are not subject to the problems of conformational averaging observed with slower NMR experiments. FET has been used to investigate the direct consequences of glycosylation by comparing interfluorophore distances in peptides and the corresponding glycopeptides.[7] For example, FET studies on the glycosylated

and non-glycosylated sequence Ac-Orn(δDns)-Ala-Val-Pro-Asn-Gly-Thr-Trp-Val-NH_2 (**14**) (based on residues A19–26 of hemagglutinin[86]) revealed the presence of two ensembles of populations with distinct interfluorophore distances. For this peptide, a distribution of conformers, centered around an interfluorophore distance of 15 Å, was observed. Upon glycosylation with a chitobiosyl disaccharide, this average interfluorophore distance was reduced to 9.5 Å (Fig. 8). Glycosylation appeared to induce the formation of a more compact structure which approximates the conformation of the glycopeptide within the native protein. This result implies that the glycosylation event has a direct impact on local peptide conformation and as it is a co-translational process, may play a distinct role in the process of protein folding.

8. Conclusions

The purification of oligosaccharyl transferase is well underway and initial characterization of several of the subunits has been accomplished. The ultimate goal of these programs is to overexpress the enzyme to obtain an ample supply of material for further studies. Access to each of the genes encoding the different subunits of OT will allow for further characterization of the enzyme complex, including an investigation into the precise role of each of the subunits. The range of techniques and experiments utilized thus far has been limited by the availability of the enzyme, however a great deal of knowledge has been gained in recent years on the mechanism of action of OT. The Asx-turn effectively addresses the precise reactivity of the NXT/S sequence by placing the required hydroxyl group within direct hydrogen-bonding distance of the carboxamide side chain of the asparagine. This conformation has been shown to be the recognition motif for OT and its hydrogen-bonding array has been incorporated into a mechanistic proposal which, through the tautomerization of the carboxamide side chain, accounts for the enhanced nucleophilicity of the asparagine residue. Analysis of the enzymatic mechanism through the use of unnatural tripeptide analogs of the NXT/S consensus sequence further support this mechanistic proposal.

The current understanding of the mechanism of action of oligosaccharyl transferase explains why nature has selected asparagine as a target for glycosylation, when proteins contain many residues which are inherently more reactive. The selection of asparagine is advantageous since aberrant glycosylation of other amino acids, such as cysteine, or aspartic acid, would generate glycoprotein products which would be insufficiently stable under physiological conditions and thereby unsuitable for the diverse functions required of these biomolecules. The unique ability of asparagine to adopt an Asx-turn provides the enzyme with a readily available mechanism through which asparagine may be distinguished from other less acceptable amino acids. The precise recognition machinery of the OT active site prohibits the glycosylation of peptides which do not contain the elements necessary for an Asx-turn. Thus, nature has evolved an enzyme which targets asparagine for glycosylation in a manner which precludes all other amino acids and generates stable and functional glycoproteins.

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