Investigation of the Yeast Oligosaccharyl Transferase

By

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B.S., Biochemistry and Cell Biology, Bucknell University, 1997

Submitted to the Department of Chemistry
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Degree of Doctor of Philosophy

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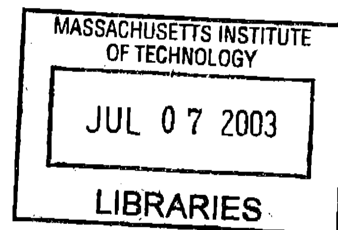

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ABSTRACT

Oligosaccharyl transferase catalyzes the first commited step in N-linked glycosylation. The enzyme complex from *Saccharomyces cerevisiae* includes nine membrane bound proteins located in the lipid bilayer of the endoplasmic reticulum, five of these proteins are essential for cell viability. The enzyme catalyzes the transfer of a tetradecasaccharide ($Glc_3Man_9GlcNAc_2$) cotranslationally from a dolichyl pyrophosphate donor to selected asparagine residues within the consensus sequence Asn-Xaa-Ser/Thr, where Xaa is any residue except for proline.

Previous attempts to isolate the enzyme have been limited to low-yield purifications from the native species. The goal of this research was to express and purify the individual essential subunits from oligosaccharyl transferase in a heterologous expression system in order to reconstitute activity in an artificial membrane system with the long term goal of establishing the minimal catalytic complex.

The four essential membrane proteins, Nlt1p, Ost2p, Swp1p and Wbp1p were expressed and analyzed in both the methylotropic yeast *Pichia pastoris* and the lytic baculovirus/insect cell expression system. The results from *P. pastoris* suggested that the protein was not well behaved and expressed at low levels. Expression of the native membrane proteins in baculovirus-infected insect cells proved more successful as the proteins were expressed to a high concentration and were readily solubilized using non-ionic detergents. However, these four proteins did not appear to be sufficient for catalytic activity. Purification of a Stt3p-Protein A conjugate suggests that this protein is required for catalysis.

Three soluble domains of key subunits from the yeast oligosaccharyl transferase were expressed in baculovirus-infected insect cells with a partial biophysical characterization of these proteins. The subunits have been determined to be monomeric in solution, heterogeneously glycosylated and structured. In addition, an interaction between two of these proteins sNlt1p and sSwp1p was established using both circular dichroism and gel filtration chromatography.

Thesis Advisor: Barbara Imperiali
Title: Ellen Swallow Richards Professor of Chemistry

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The life of a scientist is molded by the people with whom he works and I am, of course, no exception. From my first science fair project, "The Effect of Time and Temperature on Liquid Dairy Products," to the culmination of that scientific collaboration: "First-order Free Radical Oxidation Kinetics of Tryptophan Induced By Transition Metals" my father introduced me to science and was always adamant about letting the data tell the story.

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List of Abbreviations

Standard one and three letter codes are used for naturally occurring amino acids.

AEBSF	4(2-Aminoethyl)benzenesulfonyl fluoride
Bn	Benzyl
Bpa	*p*-benzoylphenylalanine
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
GlcNAc	N-Acetylglucosamine
HEPES	N-(2-Hydroxyethyl)piperazine-N'-2(2-ethanesulfonic acid
HPLC	High Performance Liquid Chromatography
MALDI	Matrix assisted laser desorption ionization
min	minute (s)
MS	Mass spectrometry
OT	Oligosaccharyl transferase
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis

TFA	Trifluoroacetic acid
Tris	Tris(hydroxynethyl)aminomethane
UV	Ultra violet

Chapter 1 Overview of Oligosaccharyl Transferase and Congenital Disorders of Glycosylation

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I-1 Introduction

Eukaryotic cells are stringently regulated and controlled through the compartmentalization of cellular processes including cell cycle control (nucleus), energy production (mitochondrion or chloroplast), protein processing [endoplasmic reticulum (ER) and Golgi apparatus (GA)] and waste removal (lysosome). Protein processing in the secretory pathway is essential for proteins destined to be released from the cell or integrated into cellular membranes. Greater than seventy percent of all proteins that are processed by these organelles are glycosylated [1, 2]. The complexity of the oligosaccharides found decorating glycoproteins is astonishing when compared to other cellular constituents, such as RNA and protein, due to the variety of chemical linkages that can be formed to a single saccharide building block. In keeping with this molecular diversity, the population of carbohydrate structures displayed by any one protein is not homogenous, thus adding to the complexity of glycoprotein structures.

In *N*-linked glycosylation, oligosaccharyl transferase (OT, EC 2.4.1.119; also known as: dolichyl-diphosphooligosaccharide-protein glycosyltransferase)

carries out the transfer of a tetradecasaccharide ($GlcNAc_2Man_9Glc_3$) from a dolichol pyrophosphate donor to selected asparagine side chains (in an Asn-Xaa-Ser/Thr consensus sequence, where Xaa is any residue except for proline) within nascent polypeptides in the lumen of the endoplasmic reticulum (Figure 1).

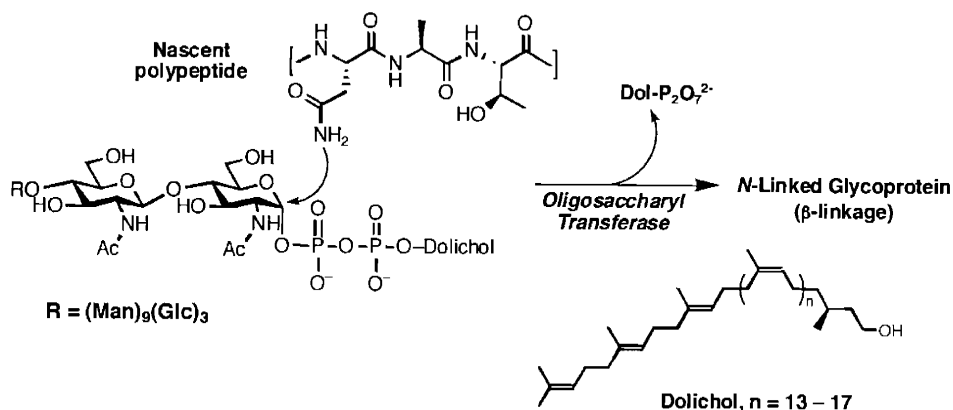


Figure I-1. Reaction catalyzed by oligosaccharyl transferase.

This chapter will present the molecular machinery that is involved in generating a nascent polypeptide and a glycosyl donor in the lumen of the ER where oligosaccharyl transferase (OT), the "gatekeeper" to the secretory pathway, effects the initial step in the biosynthesis of *N*-linked glycoproteins. The individual proteins in the OT complex and homologous proteins across species will be presented. In addition, diseases which arise from mutations in the asparagine-linked glycosylation (ALG) or dolichol pathway will be addressed.

I-2 Protein Processing Machinery and the Generation of Nascent Polypeptides in the Endoplasmic Reticulum

In order to appreciate the complexity of the oligosaccharyl transferase-catalyzed process, one must first understand the context within which the enzyme operates (Figure 2). Proteins destined for the secretory pathway are synthesized with a signal sequence that is recognized by the signal recognition particle (SRP) on the cytosolic surface of the ER membrane during translation. Following recognition by the SRP, the polypeptide is transferred to the translocon machinery which carries the polypeptide across the ER membrane [3-5]. Studies focusing on the signal peptidase complex, which cleaves the signal sequence from the polypeptide in the ER after translocation, have suggested that Sec11p is critical for catalysis in yeast [6]. During translocation, the nascent protein is directed towards OT. Nascent proteins have been shown to be glycosylated only once the Asn-Xaa-Thr/Ser consensus sequence for *N*-linked glycosylation is at least 30-40 Å from the face of the ER membrane [7].

Each of the above processes is highly interdependent. For example, a study has examined the relationship between the signal peptidase and OT. Inhibition of signal peptidase activity decreases the efficiency of glycosylation near the signal sequence. It has been proposed that the hydrophobic character of the signal sequence is able to direct the *N*-terminus of the protein into the ER membrane and then away from the active site of OT [8].

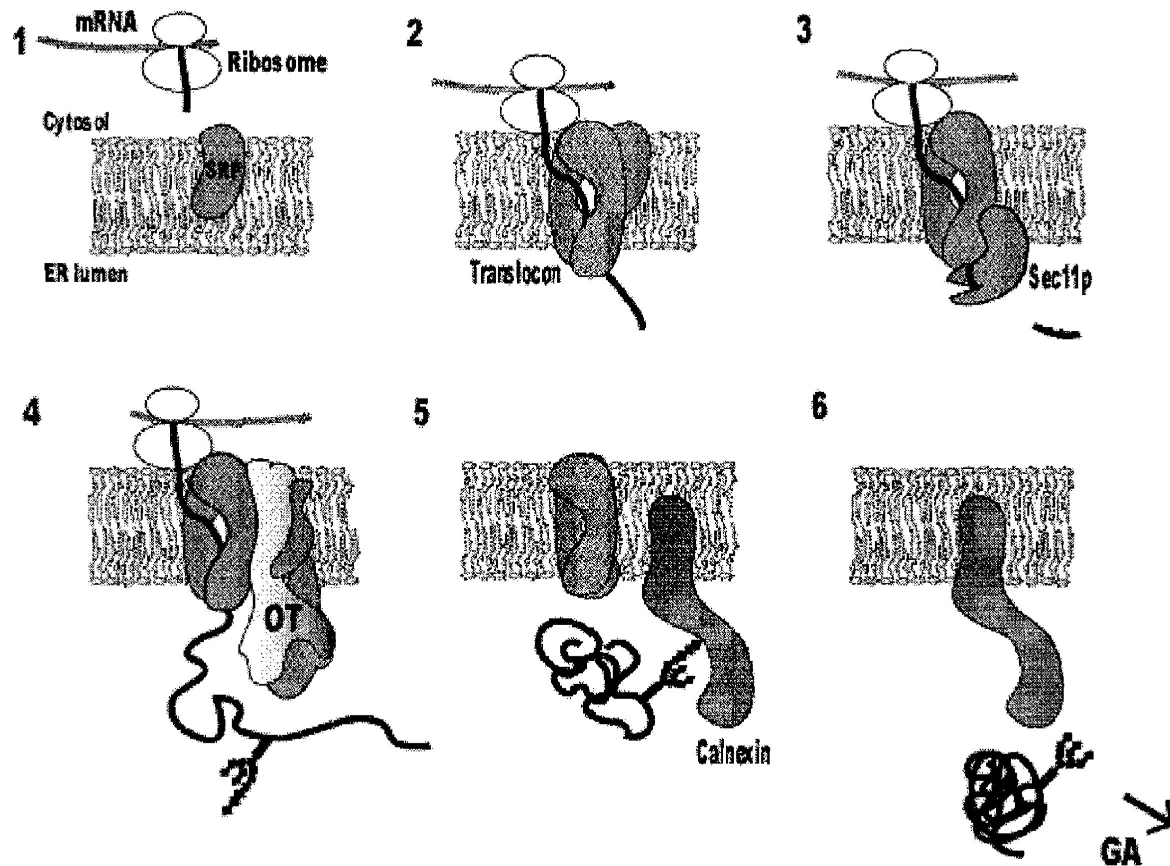


Figure I-2. Macromolecular machinery associated with the ER membrane. **1.** Translation of signal peptide and interaction of ribosome with SRP. **2.** Translocation of nascent peptide into ER. **3.** Signal peptide cleavage by Sec11p. **4.** Glycosylation of nascent polypeptide by oligosaccharyl transferase. **5.** Interaction of partially folded nascent glycoproteins with chaperones. **6.** Release of folded glycosylated proteins to the secretory pathway.

Protein chaperones are also critical to protein processing in the ER. Calnexin is a membrane-bound chaperone which is also located within this machinery in yeast [9, 10]. This protein binds to partially glucosylated and unfolded glycoproteins generated by OT in the ER, facilitating proper folding by protein-carbohydrate interactions. Similarly, calreticulin is a soluble protein that inhibits protein oligomerization and carbohydrate degradation to produce a correctly folded protein [11].

I-3 Assembly and Reactivity of Complex Dolichol Pyrophosphate-Linked Glycosyl Donors

The glycosyl donor for oligosaccharyl transferase is a tetradecasaccharide α-linked at the reducing terminus to dolichol pyrophosphate (Dol-PP-$GlcNAc_2Man_9Glc_3$). The oligosaccharide donor is biosynthesized first by the transfer of GlcNAc-P to Dol-P followed by the stepwise addition of monosaccharides to Dol-PP-GlcNAc. The transformations are catalyzed by a series of enzymes that comprise the dolichol pathway [12]. In *S. cerevisiae* the dolichol pathway enzymes are collectively termed the ALG family of enzymes (Figure 3). Several of the enzymes in the dolichol pathway have been identified. In the biosynthesis of the glycosyl donor, the first seven monosaccharides are transferred to the dolichol pyrophosphate on the cytoplasmic face of the ER membrane using the activated glycosyl donors, UDP-GlcNAc and GDP-Man. At this point, the oligosaccharide is translocated by the yeast "flippase" Rft1p into the lumen of the ER. Active transport is required to flip the carbohydrate chain across the ER membrane due to the hydrophilicity of the carbohydrates. The mechanism by which Rft1p acts is currently unknown, although the activity of the flippase appears to be ATP-independent. Rft1p is highly conserved among species that effect *N*-linked glycosylation [13].

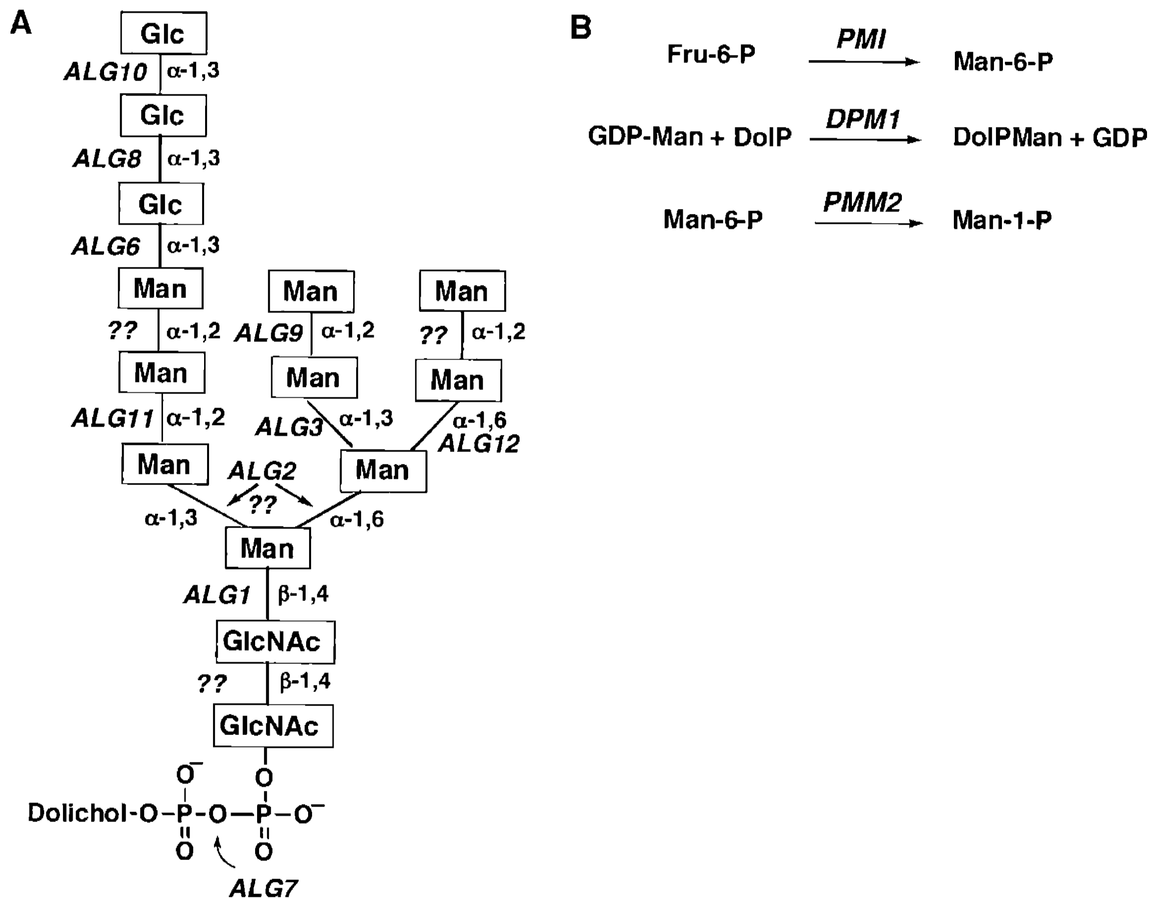


Figure I-3. The biosynthetic pathway of the N-linked glycosylation substrate. A. The fully assembled product of the dolichol pathway illustrating the known *S. cerevisiae* genes for bond constructions; B. Additional genes known to be required for synthesis of monosaccharide building blocks.

Following translocation of the oligosaccharide across the ER membrane, the remaining monosaccharides are transferred to the dolichol pyrophosphate substrate from Dol-P-Man and Dol-P-Glc activated saccharide donors [12]. The full-length oligosaccharide is then available to OT for *N*-linked glycosylation.

Gilmore and co-workers have examined the carbohydrate preference of OT with the following substrates: Dol-PP-$GlcNAc_2Man_9Glc_3$, Dol-PP-$GlcNAc_2Man_9Glc_2$, Dol-PP-$GlcNAc_2Man_9Glc$ and Dol-PP-$GlcNAc_2Man_9$. It was

determined that OT shows a preference for the full-length oligosaccharide substrate and has no preference amongst the remaining glycosyl donors [14, 15]. In this study, the authors also proposed both a regulatory and catalytic site for the oligosaccharide bound to OT. In addition to the oligosaccharides mentioned above, Cacan and co-workers identified a Chinese hamster ovary cell line which produces the truncated oligosaccharide substrates, Dol-PP-$GlcNAc_2Man_5Glc_{(0-3)}$. In studies examining substrate specificity, these truncated oligosaccharides could be transferred to proteins and subsequently processed by deglucosylating and demannosylating enzymes [16, 17]. While glycosylation is highly conserved, there are significant differences in glycosyl donors in different species. Unlike mammalian, plant and insect cells which utilize the same oligosaccharide substrate, trypanosomatids catalyze the transfer of carbohydrates lacking the terminal glucose residues and these carbohydrates contain 6, 7 or 9 mannose units [18]. The lack of terminal glucose residues on the oligosaccharides is due to the fact that trypanosomatids do not synthesize the glucose donor, Dol-P-Glc [19, 20].

An important role of the enzymes in the Golgi apparatus is to trim and elaborate the oligosaccharides in the nascent *N*-linked glycoproteins generated in the ER. While the initial OT-catalyzed protein modification in the ER is highly conserved, different species encode different processing enzymes so that the final protein glycoconjugates vary considerably from species to species (Figure I-4) [21].

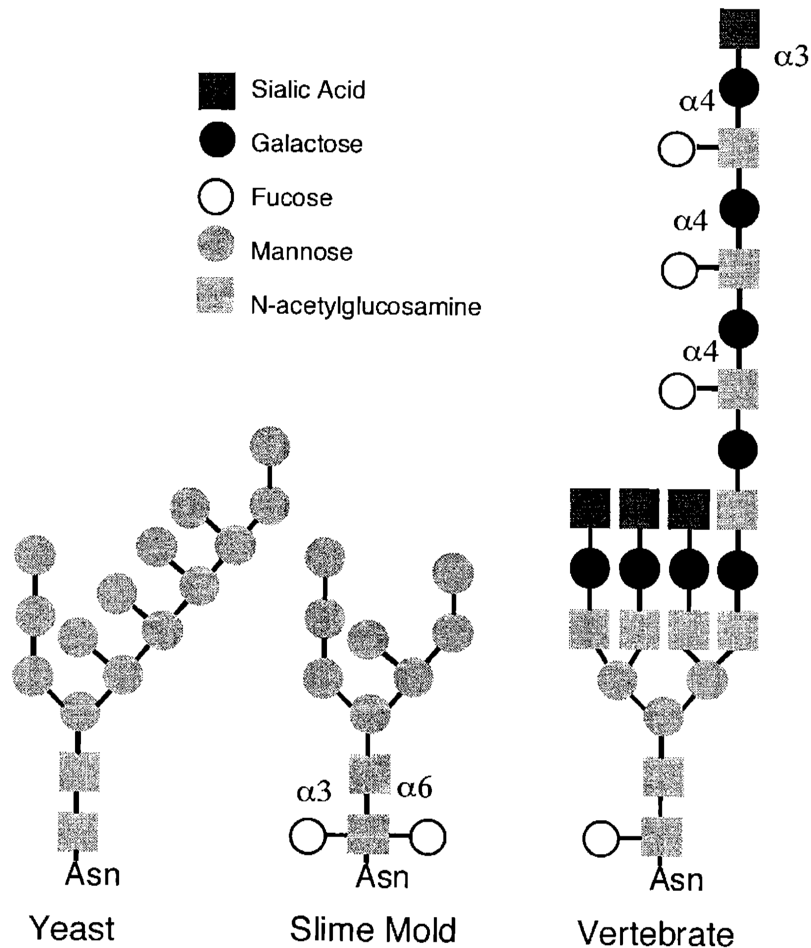


Figure I-4. Selected examples of glycoproteins processed by the Golgi Apparatus illustrating species variation.

Extensive research has probed the optimal peptide sequence for oligosaccharyl transferase. The consensus sequence was originally determined to be –Asn-Xaa-Thr/Ser-, where Xaa is any residue except for proline [22, 23]. Sequence and subunit analysis on *in vitro* glycosylation identified the enhanced consensus sequence –Asn-Xaa-Thr/Ser-Val-Thr- [1, 24, 25]. Further experiments have identified a cyclized structure which constrains the polypeptide

into an "Asx-turn." This conformation promotes tight binding of the polypeptide substrate to the enzyme [26].

As a result of these experiments, it has been proposed that an active site base abstracts a proton from the nitrogen amide of the asparagine side chain which subsequently forms an imidate tautomer, which as a functional nucleophile, attacks the oligosaccharide substrate during catalysis (Figure 1-5) [27].

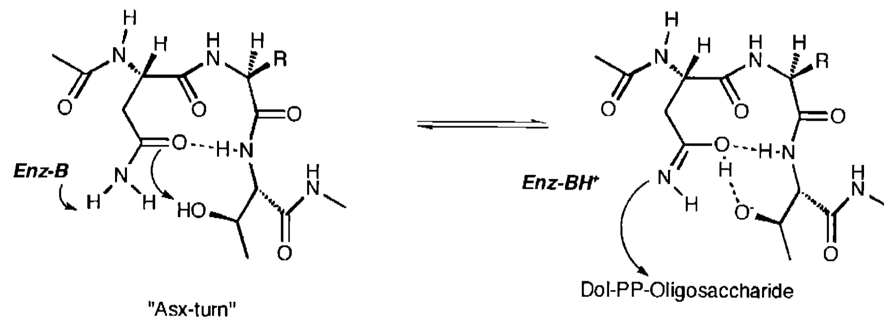


Figure I-5. Proposed mechanism of action for yeast oligosaccharyl transferase.

I-4 The Composition of the Oligosaccharyl Transferase Complex

Oligosaccharyl transferase is a multimeric protein complex wherein many of the proteins are highly homologous, from protozoan to mammalian species. The best studied OT complex is that derived from the budding yeast, *Saccharomyces cerevisiae.* This complex includes nine different membrane-bound subunits (**Ost1p**, **Ost2p**, Ost3p, Ost4p, Ost5p, Ost6p, **Stt3p**, **Swp1p** and **Wbp1p**), five (in bold) of which have been determined to be essential for cell

viability (Figure 1-6) [28-32]. Deletions of the non-essential proteins result in a marked underglycosylation or reduced cell growth, although the cells are still viable [33-36]. A number of protein purification schemes have yielded complexes of four, five, six or eight subunits [28, 36-39].

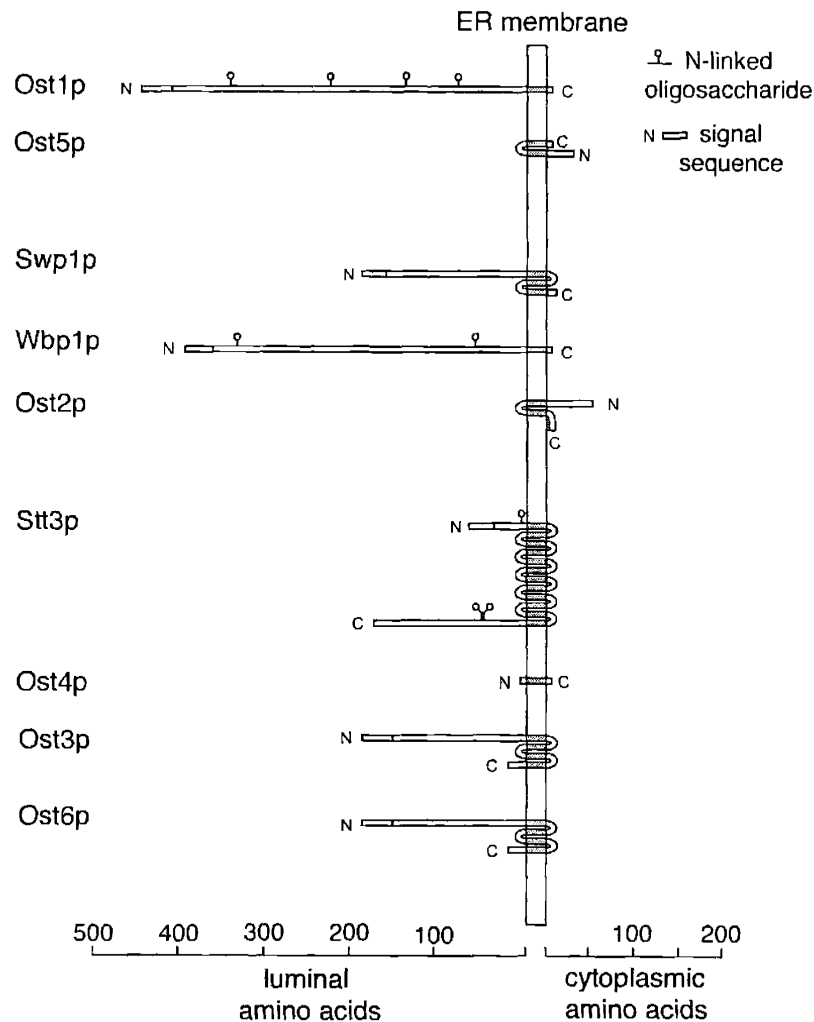


Figure I-6. Subunit composition of yeast (*S. cerevisiae*) oligosaccharyl transferase complex illustrating predicted membrane orientation. The membrane proteins are displayed according to three different subcomplexes. Subcomplex 1 consists of Wbp1p, Swp1p and Ost2p. Subcomplex 2 comprises Ost1p and Ost5p. Subcomplex 3 includes Stt3p, Ost3p and Ost4p. Reproduced from Knauer and Lehle [40].

Genetic, immunoprecipitation and over-expression experiments have revealed that the OT complex is assembled from three subcomplexes [29-31, 41, 42]. The first subcomplex includes Wbp1p, Swp1p and Ost2p, the second subcomplex includes Ost1p and Ost5p, while the final subcomplex consists of Stt3p, Ost3p and Ost4p [43]. In addition to the subcomplexes mentioned above, proteins within different subcomplexes have been shown to interact with each other [44, 45].

Yeast (*S. cerevisiae*)	Mammalian (*H. sapiens*)	Homology Details
Nlt1p (64/62 kDa glycoforms)	**Ribophorin I** (65 kDa)	Yeast subunit 28% identity (58% similarity) with Ribophorin I
Swp1p (30 kDa)	**Ribophorin II** (65 kDa)	Yeast subunit 22% identity (49% similarity) with C-terminus of Ribophorin II
Wbp1p (45 kDa)	**OST48** (50 kDa)	Yeast subunit 25% identity (50% similarity with OST48
Ost2p (15 kDa)	**DAD1** (12.5 kDa)	Yeast subunit 45% identity (68% similarity) with DAD1 (Ost2p extended at the N-terminus)
Ost3p	Not known	
Ost4p	Not known	
Ost5p	Not known	
Ost6p	Not known	
Stt3p (82 kDa)	**Stt3p** (80 kDa)	Yeast subunit 51% identity (65% similarity) with human homolog

Table I-1. Comparison of Mammalian and Yeast OT Complexes.

In the first subcomplex, Wbp1p was named for its ability to bind wheat germ agglutinin, which is indicative of a glycosylated protein [32]. The protein

includes two potential *N*-linked glycosylation sites, which are both utilized, and a single membrane-spanning domain. The yeast protein shares 25% identity and 50% similarity with the mammalian homolog Ost48 (Table 1) [40]. This protein has been implicated in binding the dolichyl-diphosphooligosaccharide substrate. Multiple cysteine-modifying reagents, including methyl methanethiolsulfonate and S-[N-biotinoylamino)ethyl]methanethiol-sulfonate (BMTS) selectively labeled Wbp1p, while preincubation with the oligosaccharide substrate prevented inactivation by BMTS [46]. Although the protein Nlt1p has not been identified as part of this subcomplex, te Heesen and colleagues utilized a split-ubiquitin system to demonstrate an interaction between Nlt1p and Wbp1p [45]. The second protein in this subcomplex, Swp1p, is also essential and was initially isolated as an allele specific suppressor of a Wbp1p mutation [31]. The protein has a molecular weight of 30 kDa and includes three transmembrane domains as determined by hydropathy analysis [31]. This protein is homologous to the mammalian Ribophorin II. Protein-crosslinking initially identified an interaction between Swp1p and Wbp1p [31]. Ost2p, the third protein of the subcomplex, is the smallest of the five essential proteins, with a mass of 16 kDa. It is also the most hydrophobic, with two or three transmembrane domains and a hydrophobic region at the *C*-terminus of the protein [29]. Ost2p is similar (45% identity) to the eukaryotic protein Dad1, which is essential in mammalian species. A temperature sensitive strain of Dad1 in the golden hamster cell line tsBN7, was shown to enter apoptosis due to the loss of Dad1 [47]. In addition, despite the

high identity between Dad1 and Ost2p, no complementation is seen when Ost2p is expressed in temperature sensitive mutants of Dad1 [47].

The next subcomplex includes Ost1p (Nlt1p) and Ost5p [28, 48]. Ost1p has a molecular weight of 64 kDa, contains four potential glycosylation sites and has 28% identity and 58% similarity to the mammalian homolog Ribophorin I. It has been observed as a 62/64 kDa doublet upon SDS-PAGE analysis in a variety of reports, indicative of different glycoforms of the enzyme. Using the ^{125}I-labeled tripeptide Asn-Bpa-Thr (Bpa: *p*-benzoylphenylalanine), Lennarz and co-workers probed the peptide binding site of OT. This peptide was a competent substrate. When irradiated, the excited state of the benzophenone moiety labeled Ost1p, suggesting that this protein subunit recognizes the polypeptide during catalysis [43, 49].

Ost5p is a 9 kDa protein with two proposed transmembrane domains. The absence of this protein is not lethal, nor does it exhibit a slowed growth rate, however, it does result in decreased glycosylation efficiency. In addition, the enzyme lacking this subunit is active *in vitro*.

The third subcomplex includes Stt3p, Ost3p and Ost4p. Stt3p is the largest of the essential proteins in the complex and was originally isolated through screening for staurosporine-supersensitive mutants as staurosporine is an inhibitor of protein kinase C [50]. Stt3p is predicted to include 12 transmembrane domains and is highly homologous to proteins found in eurkaryotic, archaebacteria and eubacteria. This protein appears to be the most

conserved protein across species. Highly homologous proteins have been cloned from *E. histolytica, C. jejuni, D. melanogaster* and *H. sapiens* [51, 52]. Interestingly, *Entamoeba histolytic* is a protozoan parasite and thus demonstrates that this protein is highly conserved through a number of branches on the evolutionary tree. More recently, the oligosaccharide modifying *N*-linked glycoproteins from *C. jejuni*, a gram-negative bacterium, has been identified as a heptasaccharide ($GlcGalNAc_5Bac$, where Bac is bacillosamine or 2,4-diacetamido-2,4,6-trideoxyglucopyranose) [53]. Protein glycosylation in this organism was eliminated with the loss of Pg1B, a membrane spanning protein, which is homologous to Stt3p, suggesting that this protein is critical to OT catalysis [54]. Further, Lennarz and co-workers made block mutations of the yeast Stt3p and observed a loss of enzymatic activity that was concurrent with the mutation of the entire 516-520WWDYG consensus sequence to alanine residues [55]. Subsequent mutagenesis of residues W517Y and D518E resulted in a lethal phenotype suggesting that these residues are critical to the proper presentation of substrate to the enzyme or are critical for catalysis and that subtle changes on the aromatic ring or length of side chain, respectively, result in dramatic phenotypic changes.

Ost3p is a 34 kDa protein. The phenotype of the null mutant yeast strain of this protein does not exhibit a slowed growth rate, although there is a differential rate of underglycosylation between 13% to 79% depending on the protein studied [33]. Ost4p is the smallest of the proteins associated with this

enzyme at 36 residues, with the majority of these residues predicted to be within the ER membrane. This protein was mutated to examine the effect different residues have on the association of this protein with Stt3p and Ost3p. Mutation of residues within the ER, and proximal to the lumen, to ionizable amino acids inhibited enzymatic activity. These mutations also inhibited the association of the subcomplex Ost3p, Ost4p and Stt3p. In contrast, point mutations at other sites in the protein including both termini and the transmembrane domain did not alter the activity of the enzyme nor did it affect the association of the complex [56]. These data suggest that the luminal domain of these subunits are interacting with each other during catalysis which is consistent with the proposed location of the catalytic site of OT in the ER lumen.

Ost6p, a 32 kDa protein that is homologous to Ost3p, is the final membrane bound protein associated with oligosaccharyl transferase [36]. Deletion of Ost6p results in a minor decrease in efficiency of glycosylation *in vitro*. The double deletion mutant of Ost6p and Ost3p results in no change in growth rate, however there is a severe level of underglycosylation. In addition, overexpression of Ost6p in Ost3p null cells restores underglycosylation suggesting that these two proteins play similar roles in the yeast OT complex.

I-5 Diseases Associated with N-linked Glycosylation

As described earlier, deletions of the essential OT subunits result in a lethal phenotype. In addition, it has been shown that deletion of UDP-GlcNAc: dolichol phosphate *N*-acetylglucosamine-1-phosphate transferase, which catalyzes the transfer of P-GlcNAc onto dolichol-P in embryonic cells results in a lethal phenotype [57]. This is further evidence that glycosylation is essential for viable cells.

In contrast, mutations to enzymes associated with the assembly or processing of *N*-linked carbohydrates have been shown to result in diseases that have broad systemic effects including physchomotor retardation, nervous system deficiencies, coagulation disorders and dysmorphic features [58]. These diseases are collectively termed congenital disorders of glycosylation (CDGs) of which there are two types. Type I CDGs (CDG1) are associated with mutations that affect the assembly of the tetradecasaccharide on the dolichol pyrophosphate donor while CDG2 diseases arise from mutations in proteins which affect *N*-linked processing.

A total of six CDG1 defects have been identified. Because of the broad specificity of oligosaccharyl transferase for the carbohydrate substrate, the truncated oligosaccharides that are formed can still be transferred onto a polypeptide chain.

Three CDGI mutations are associated with proteins involved in the synthesis of the tetradecasaccharide donor. Mutations in *ALG3* show a decreased transfer of Man from Dol-P-Man to Dol-PP-GlcNAc$_2$Man$_5$ (Figure I-3A). Mutations in *ALG12* affect the transfer of Man from Dol-P-Man to Dol-PP-GlcNAc$_2$Man$_7$ and mutations in *ALG6* inhibit the transfer of Glc from Dol-P-Glc to Dol-PP-GlcNAc$_2$Man$_9$. The most recently identified CDGI involves *ALG12* [59]. A mutation to this gene results in the truncated oligosaccharide substrate, Dol-PP-GlcNAc$_2$Man$_7$. The phenotype of a single nucleotide mutant from *ALG12* is under-glycosylated serum glycoproteins, which result in peripheral and central nervous system abnormalities [60].

Three additional CDG1 diseases are associated with the synthesis of the monosaccharide building blocks (Figure I-3B). These diseases result from alterations in the enzymes which catalyze the transfer of Man from GDP-Man to Dol-P-Man [dolicyl-phosphate-mannose synthetase (*DPM1*)], the conversion of Man-6-P to Man-1-P [phosphomannomutase (*PMM2*)] and the conversion of Fru-6-P to Man-6-P [phosphomannose isomerase (*PMI*)]. The most common CDGI is associated with *PMM2*, which is lethal with some homozygous mutations [61, 62]. Viable mutations still have significant mortality after one year (20%) and the phenotypic mutations are extensive and include severe retardation [63]. In addition, over sixty point mutations have been identified in the PMM2 gene which result in a disease state [64].

I-6 Dissertation Objective

Oligosaccharyl transferase is an integral part of the macromolecular machinery that is critical for the proper functioning of all eukaryotic cells. Research in the past decade has elucidated the cellular constituents responsible for *N*-linked glycosylation and the related machinery that provides and processes the substrates for the oligosaccharyl transferase. The focus of the research in this dissertation is to further probe the proteins involved in activity of the enzyme and to examine protein-protein interactions within the complex.

Chapter II describes efforts towards the expression, purification and characterization of the essential membrane proteins of this complex. Expression systems utilized includes *P. pastoris* and the lytic baculovirus/insect cell systems. In addition, further experiments were initiated to introduce a more sensitive assay to probe the activity of the purified proteins. Chapter III describes the research which produced three soluble domains of the essential membrane proteins. These proteins have been determined to be well-behaved and to interact with each other. Chapter IV describes a summary of the dissertation and future directions for this project.

I-7 References

1. Gavel, Y. & von Heijne, G. (1990) Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: Implications for protein engineering, *Protein Eng. 3*, 433-442.

2. Mononen, I. & Karjalainen, E. (1999) Structural comparison of protein sequences around potential N-glycosylation sites, *Biochim. Biophys. Acta. 788*, 364-367.

3. Johnson, A. & van Waes, M. (1999) The translocon: A dynamic gateway at the ER membrane, *Annu. Rev. Cell Dev. Biol. 19*, 87-119.

4. Young, B., Craven, R., Reid, P., Willer, M. & Stirling, C. (2001) Sec63p and Far2p are required for the translocation of SRP-dependent precursors into the yeast endoplasmic reticulum *in vivo*, *EMBO J. 20*, 262-271.

5. Potter, M. & Nicchitta, C. (2002) Endoplasmic reticulum-bound ribosomes reside in stable association with the translocon following termination of protein synthesis, *J. Biol. Chem. 277*, 23314-23220.

6. Van Valkenburgh, C., Chen, X., Mullins, C., Fang, H. & Green, N. (1999) The catalytic mechanism of endoplasmic reticulum signal peptidase appears to be distinct from most eubacterial signal peptidases, *J. Biol. Chem. 274*, 11519-11525.

7. Nilsson, I. & von Heijne, G. (1993) Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane, *J. Biol. Chem. 268*, 5798-5801.

8. Chen, X., Van Valkenburgh, C., Liang, H., Fang, H. & Green, N. (2001) Signal peptidase and oligosaccharyltransferase interact in a sequential and dependent manner within the endoplamic reticulum, *J. Biol. Chem. 276*, 2411-2416.

9. Parodi, A. (1999) Reglucosylation of glycoproteins and quality control of glycoprotein folding in the endoplasmic reticulum of yeast cells, *Biochim. Biophys. Acta. 1426*, 287-295.

10. Schrag, J., Procopio, D., Cygler, M., Thomas, D. & Bergeron, J. (2003) Lectin control of protein folding and sorting in the secretory pathway, *Trends Biochem. Sci. 28*, 49-57.

11. Ellgaard, L. & Helenius, A. (2001) ER quality control: Towards an understanding at the molecular level, *Curr. Opin. Cell Biol. 13*, 431-437.

12. Burda, P. & Aebi, M. (1999) The dolichol pathway of N-linked glycosylation, *Biochim. Biophys. Acta. 1426*, 239-257.

13. Helenius, J., Ng, D., Marolda, C., Walter, P., Valvano, M. & Aebi, M. (2002) Translocation of lipid-linked oligosaccharides across the ER membrane requires Rft1 protein, *Nature. 415*, 447-450.

14. Kelleher, D., Karaoglu, D. & Gilmore, R. (2001) Large-scale isolation of dolichol-linked oligosaccharides with homogeneous oligosaccharide structures:

Determination of steady-state dolichol-linked oligosaccharide compositions, *Glycobiology. 11*, 321-333.

15. Kararoglu, D., Kelleher, D. & Gilmore, R. (2001) Allosteric regulation provides a molecular mechanism for preferential utilization of the fully assembled dolichol-linked oligosaccharide by the yeast oligosaccharyltransferase, *Biochemistry. 40*, 12193-12206.

16. Duvet, S., Chirat, F., Mit, A., Verbert, A., Dubuisson, J. & Cacan, R. (2000) Reciprocal relationship between alpha 1,2 mannosidase processing and reglucosylation in the rough endoplasmic reticulum of Man-P-Dol deficient cells, *Eur. J. Biochem. 267*, 1146-1152.

17. Foulquier, F., Harduin-Lepers, A., Duvet, S., Marchal, I., Mir, A., Delannoy, P., Chirat, F. & Cacan, R. (2002) The unfolded protein response in a dolichyl phosphate mannose-deficient Chinese hamster ovary cell line points out the key role of a demannosylation step in the quality-control mechanism of N-glycoproteins, *Biochem. J. 362*, 491-498.

18. Bosch, M., Trometta, S., Engstrom, U. & Parodi, A. (1988) Characterization of dolichol diphosphate oligosaccharide: Protein oligosaccharyltransferase and glycoprotein-processing glucosidases occurring in trypanosomatid protozoa, *J. Biol. Chem. 263*, 17360-17365.

19. Parodi, A. (1993) N-Glycosylation in trypanosomatid protozoa, *Glycobiology. 3*, 193-199.

20. de la Canal, L. & Parodi, A. (1987) Synthesis of dolichol derivatives in trypanosomatids, *J. Biol. Chem. 262,* 11128-11133.

21. Roth, J. (2002) Protein N-glycosylation along the secretory pathway: Relationship to organelle topography and function, protein quality control, and cell interactions, *Chem. Rev. 102*, 285-304.

22. Imperiali, B. & Shannon, K. (1991) Differences between Asn-Xaa-Thr containing peptides-A comparison of solution conformation and substrate behavior with oligosaccharyltransferase, *Biochemistry. 30*, 4374-4380.

23. Marshall, R. (1974) The nature and metabolism of the carbohydrate-peptide linkages of glycoproteins, *Biochem. Soc. Symp. 40*, 17-26.

24. Imperiali, B., Spencer, J. & Struthers, M. (1994) Structural and functional characterization of a constrained Asx-turn motif, *J. Am. Chem. Soc. 116*, 8424-8425.

25. Imperiali, B. & O'Connor, S. (1998) The conformational basis of asparagine-linked glycosylation, *Pure Appl. Chem. 70*, 33-40.

26. Imperiali, B., Shannon, K. & Rickert, K. (1992) Role of peptide conformation in asparagine-linked glycosylation, *J. Am. Chem. Soc. 114*, 7942-7944.

27. Imperiali, B., Shannon, K., Unno, M. & Rickert, K. (1992) A mechanistic proposal for asparagine-linked glycosylation, *J. Am. Chem. Soc. 114*, 7944-7945.

28. Pathak, R., Parker, C. & Imperiali, B. (1995) The essential NLT1 gene encodes the 64 kDa glycoprotein subunit of the oligosaccharyl transferase., *FEBS Lett. 362*, 229-234.

29. Silberstein, S., Collins, P., Kelleher, D. & Gilmore, R. (1995) The essential Ost2 gene encodes the 16-KD subunit of the yeast oligosaccharyltransferase, a highly conserved protein expressed in diverse eukaryotic organisms, *J. Cell Biol. 131*, 371-383.

30. Zufferey, R., Knauer, R., Burda, P., Stagljar, I., te Hessen, S., Lehle, L. & Aebi, M. (1995) Stt3p, a highly conserved protein required for yeast oligosaccharyl transferase activity *in vivo*, *EMBO J. 14*, 4949-4960.

31. te Hessen, S., Knauer, R., Lehle, L. & Aebi, M. (1993) Yeast Wbp1p and Swp1p form a protein complex essential for oligosaccharyl transferase activity, *EMBO J. 12*, 279-284.

32. te Hessen, S., Rauhut, R., Aebersold, R., Abelson, J., Aebi, M. & Clark, M. (1991) An essential 45 kDa yeast transmembrane protein reacts with anti-nuclear pore antibodies: Purification of the protein, immunolocalization and cloning of the gene, *Eur. J. Cell Biol. 56*, 8-18.

33. Karaoglu, D., Kelleher, D. & Gilmore, R. (1995) Functional characterization of Ost3p. Loss of the 34-kD subunit of the *Saccharomyces cerevisiae* oligosaccharyltransferase results in biased underglycosylation of acceptor substrates, *J. Cell Biol. 130*, 567-577.

34. Chi, J., Roos, J. & Dean, N. (1996) The Ost4 gene of *Saccharomyces cerevisiae* encodes an unusually small protein required for normal levels of oligosaccharyltransferase activity, *J. Biol. Chem. 271*, 3132-3140.

35. Reiss, G., te Hessen, S., Gilmore, R., Zufferey, R. & Aebi, M. (1997) A specific screen for oligosaccharyltransferase mutations identifies the 9 kDa OST5 protein required for optimal activity in vivo and in vitro, *EMBO J. 16*, 1164-1172.

36. Knauer, R. & Lehle, L. (1999) The oligosaccharyltransferase complex from *Saccharomyces cerevisiae*: Isolation of the Ost6p gene, its synthetic interaction with Ost3p, and analysis of the native complex, *J. Biol. Chem. 274*, 17249-17256.

37. Knauer, R. & Lehle, L. (1994) The N-Oligosaccharyltransferase complex from yeast, *FEBS Lett. 344*, 83-86.

38. Pathak, R. & Imperiali, B. (1997) A dual affinity tag on the 64-kDa Nlt1p subunit allows the rapid characterization of mutant yeast oligosaccharyl transferase complexes, *Arch. Biochem. Biophys. 338*, 1-6.

39. Kelleher, D. & Gilmore, R. (1994) The *Saccharomyces cerevisiae* oligosaccharyltransferase is a protein complex composed of Wbp1p, Swp1p, and 4 additional polypeptides, *J. Biol. Chem. 269*, 12908-12917.

40. Knauer, R. & Lehle, L. (1999) The oligosaccharyltransferase complex from yeast, *Biochim. Biophys. Acta. 1426*, 259-273.

41. Karaoglu, D., Kelleher, D. & Gilmore, R. (1997) The highly conserved Stt3p protein is a subunit of the yeast oligosaccharyltransferase and forms a subcomplex with Ost3p and Ost4p, *J. Biol. Chem. 272*, 32513-32520.

42. Spirig, U., Glaras, M., Bodmer, D., Reiss, G., Burda, P., Lippuner, V., te Hessen, S. & Aebi, M. (1997) The Stt3 protein is a component of the yeast oligosaccharyltransferase complex, *Mol. Gen. Genet. 256*, 628-637.

43. Yan, Q. & Lennarz, W. (1999) Oligosaccharyltransferase: A complex multisubunit enzyme of the endoplasmic reticulum, *Biochem. Biophys. Res. Commun. 266*, 684-689.

44. Park, H. & Lennarz, W. (2000) Evidence for interaction of yeast protein kinase C with several subunits of oligosaccharyl transferase, *Glycobiology. 10*, 737-744.

45. Stagljar, I., Korostensky, C., Johnsson, N. & te Hessen, S. (1998) A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins *in vivo, Proc. Natl. Acad. Sci. U.S.A. 95*, 5187-5192.

46. Pathak, R., Hendrickson, T. & Imperiali, B. (1995) Sulfhydryl modification of the yeast Wbp1p inhibits oligosaccharyl transferase activity, *Biochemistry. 34*, 4179-4185.

47. Makishima, T., Nakashima, T., Nagata-Kuno, K., Fukushima, K., Iida, H., Sakaguchi, M., Ikehara, Y., Komiyama, S. & Nishimoto, T. (1997) The highly conserved DAD1 protein involved in apoptosis is required for N-linked glycosylation, *Genes Cells. 2*, 129-141.

48. Silberstein, S., Collins, P., Kelleher, D., Rapiejko, P. & Gilmore, R. (1995) The a subunit of the Saccharomyces cerevisiae oligosaccharyltransferase

complex is essential for vegetative growth of yeast and is homologous to mammalian ribophorin I, *J. Cell Biol. 128*, 525-536.

49. Yan, Q., Prestwich, G. & Lennarz, W. (1999) The Ost1p subunit of yeast oligosaccharyl transferase recognizes the peptide glycosylation site sequence, -Asn-X-Ser-Thr, *J. Biol. Chem. 274*, 5021-5025.

50. Yoshida, S., Ohya, Y., Nakano, A. & Anraku, Y. (1995) STT3, a novel essential gene related to the PKC1/STT1 protein kinase pathway, is involved in protein glycosylation in yeast, *Gene. 164*, 167-172.

51. Wacker, M., Nita-Lazar, M. & Aebi, M. (2001) PglB, an oligosaccharyltransferase in the eubacterium *Campylobacter jejuni*?, *Glycobiology. 11*.

52. Gutiérrez, A., Sánchez-López, R., Ramos, M. & Alagón, A. (2000) Cloning of the *Entamoeba histolytica* STT3 gene, a subunit of the oligosaccharyltransferase complex, *Arch. Med. Res. 31*, S162-S164.

53. Young, N., Brisson, J., Kelly, J., Watson, D., Tessier, L., Lanthier, P., Jarrell, H., Cadotte, N., St. Michael, F., Aberg, E. & Szymanski, C. (2002) Structure of the N-linked glycan present on multiple glycoproteins in the gram-negative bacterium, *Campylobacter Jejuni*, *J. Biol. Chem. 277*, 42530-42539.

54. Wacker, M., Linton, D., Hitchen, P., Nita-Lazar, M., Haslam, S., North, S., Panico, M., Morris, H., Dell, A., Wren, B. & Aebi, M. (2002) N-linked glycosylation in *Campylobacter jejuni* and Its functional transfer into *E. coli*, *Science. 298*, 1790-1793.

55. Yan Q & WJ, L. (2002) Studies on the Function of Oligosaccharyl Transferase Subunits, *J. Biol. Chem. 277*, 47692-47700.

56. Kim, H., Park, H., Montalvo, L. & Lennarz, W. (2000) Studies on the role of the hydrophobic domain of Ost4p in interactions with other subunits of yeast oligosaccharyl transferase, *Proc. Natl. Acad. Sci. U.S.A. 97*, 1516-1520.

57. Marek, K., Vijay, I. & Marth, J. (1999) A recessive deletion in the GlcNAc-1-phosphotransferase gene results in periimplantation embryonic lethality, *Glycobiology. 9*, 1263-1271.

58. Matthijs, G. & Jaeken, J. (2001) Congenital disorders of glycosylation, *Annu. Rev. Genom. Hum. Genet. 2*, 129-151.

59. Burda, P., Jakob, C., Beinhauer, J., Hegemann, J. & Aebi, M. (1999) Ordered assembly of the asymmetrically branched lipid-linked oligosaccharide in the endoplasmic reticulum is ensured by the substrate specificity of the individual glycosyltransferases, *Glycobiology. 9*, 617-625.

60. Chantret, I., Dupre, T., Delenda, C., Bucher, S., Dancourt, J., Barnier, A., Charollais, A., Heron, D., Bader-Meunier, B., Danos, O., Seta, N., Durand, G., Oriol, R., Codogno, P. & Moore, S. (2002) Congenital disorders of glycosylation type Ig is defined by a deficiency in dolichyl-P-mannose:$Man_7GlcNAc_2$-PP-dolichyl mannosyltransferase, *J. Biol. Chem. 277*, 25815-25822.

61. Matthijs, G., Schollen, E., Pardon, E., Veiga Da Cunha, M., Jaeken, J., Cassiman, J. & van Schaftingen, E. (1997) Mutations in PMM2, a

phosphomannomutase gene on chromosome 16p13, in carbohydrate-deficient glycoprotein type 1 syndrome (Jaeken syndrome), *Nat. Genet. 16*, 88-92.

62. Matthijs, G., Schollen, E., Van Schaftingen, E., Cassiman, J. & Jaeken, J. (1998) Lack of homozygotes for the most frequent disease allele in carbohydrate-deficient glycoprotein syndrome type 1A, *Am. J. Hum. Genet. 62*, 542-550.

63. Tayebi, N., Andrews, D., Park, J., Orvisky, E., McReynolds, J., Sidransky, E. & Krasnewich, D. (2002) A deletion-insertion mutation in the phosphomannomutase 2 gene in an African American patient with congenital disorders of glycosylation-Ia, *Am. J. Med. Genet. 108*, 241-246.

64. Matthijs, G., Schollen, E., Bjursell, C., Erlanden, A., Freeze, H., Imtiaz, F., Kjaergaard, S., Martinsson, T., Schwartz, M., Seta, N., Vuillaumier-Barot, S., Westphal, V. & Winchester, B. (2000) Mutations in PMM2 that cause congenital disorders of glycosylation, type Ia (CDG-Ia), *Hum. Mutat. 16*, 386-394.

Chapter II: Investigation into the Reconstitution of yeast Oligosaccharyl Transferase

II-1 Introduction

The purification and reconstitution of oligosaccharyl transferase has been challenging due to the low natural abundance of the component proteins in all cells. The majority of studies aimed at understanding OT have been limited to genetic analysis, the study of peptide inhibitors to probe the mechanism of catalysis on crude or partially purified microsomal preparations and the identification of subunits involved in catalysis with photo-affinity probes [1-3]. While genetic studies are important in establishing which of the subunits are essential for activity, these experiments are of limited use in understanding the detailed structure and roles of each subunit in catalysis. Previous purification protocols have resulted in active complexes of four, five, six or eight proteins [4-8]. The use of peptide inhibitors provides important information regarding the mechanism of catalysis, but fails to yield specific structural information about the roles of individual subunits in the complex. Finally, the results of chemical cross-linking experiments have shown that more than one protein is labeled depending on the cross-linking agent utilized, suggesting close proximity of the protein subunits. Due to the low yields and limited techniques available to reveal the roles of the subunits in catalysis, the ultimate goal of the project described in this chapter is to express and purify the essential subunits of OT using a

heterologous protein expression system. The accomplishment of this goal would provide the foundation reconstitution of enzymatic activity in a form amenable to facile mutagenesis and a method to study the contribution of each subunit towards catalysis.

II-2 Expression of Native Subunits in *Pichia pastoris*

Previous research in the Imperiali group using a sequence of purification steps, including concanavalin A chromatography, Ni-NTA chromatography and purification utilizing the FLAG epitope identified five proteins which formed an active OT complex from *S. cerevisiae* (Figure II-1) [6]. Of these isolated proteins, four had been identified by genetic studies as essential for catalytic activity (Nlt1p, Ost2p, Swp1p and Wbp1p) whereas Ost3p was deemed not essential. Deletion of Ost3p from *S. cerevisiae* results in a slowed growth rate and underglycosylation, while deletion of any of the remaining four subunits eliminates cell viability [4, 9-13]. Taken together, the genetic studies and purification experiments suggested that the four proteins, Nlt1p, Ost2p, Swp1p and Wbp1p should be sufficient for catalysis.

With these results, the next objective was to obtain useful amounts of each of the proteins using a heterologous expression system for eventual reconstitution attempts. The four essential subunits, Nlt1p, Ost2p, Swp1p and Wbp1p were cloned into the pPicz (Invitrogen) *P. pastoris* expression vector, which contains an inducible AOX1 promoter for transient high-level protein

expression. Each protein contained a signal sequence to direct the protein to the secretory pathway, a poly-histidine tag and FLAG epitope for purification and a Factor Xa cleavage sequence to enable removal of the purification epitopes after purification (Figure II-2). The proteins were designed so that the poly-histidine tag and FLAG epitope were located on the cytosolic face of the endoplasmic reticulum membrane, since the active site is located in the lumen of the endoplasmic reticulum. Therefore, the affinity sequences for Nlt1p, Swp1p and Wbp1p were directed to the C-terminus of the protein and these tags were encoded to the N-terminus of Ost2p.

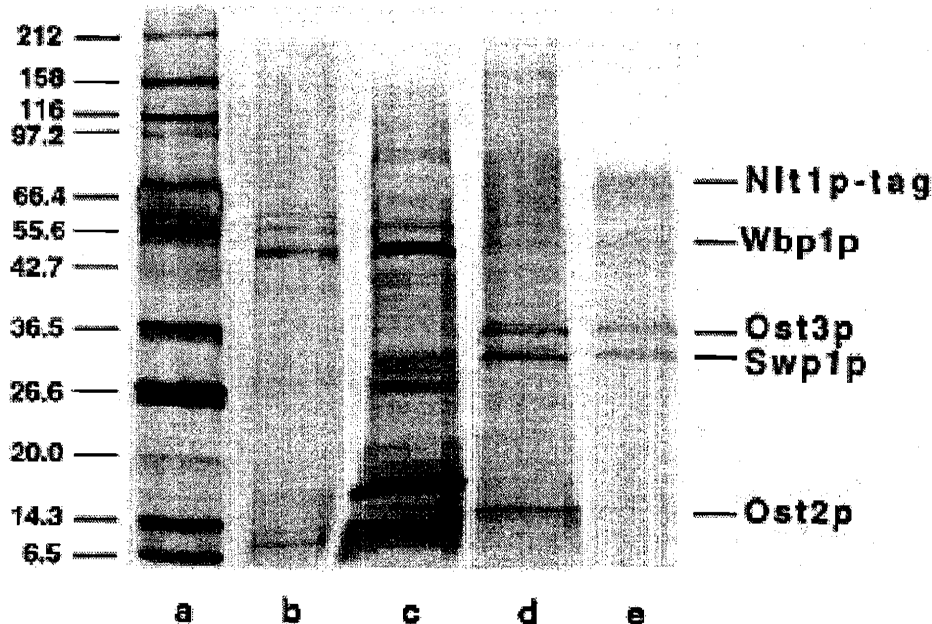


Figure II-1. Silver stain of the purification of oligosaccharyl transferase complex from *S. cerevisiae* using a poly-histidine and FLAG tag on Nlt1p. The lanes are as described below. (a) molecular weight standards, (b) solubilized yeast microsomes, (c) proteins purified using concanavalin A chromatography, (d) proteins purified using Ni-NTA chromatography and (e) proteins purified using an immobilized antibody with specificity for the FLAG epitope. Adapted from ref. [6].

The proteins were expressed in *P. pastoris* and purified using Ni-NTA. For each of the four proteins examined, only a small fraction of protein, including many degradation and/or truncation products, was solubilized with detergent (for an example with Swp1p, see Figure II-3).

Nlt1p (tag)

Ost2p (tag)

Swp1p (tag)

Wbp1p (tag)

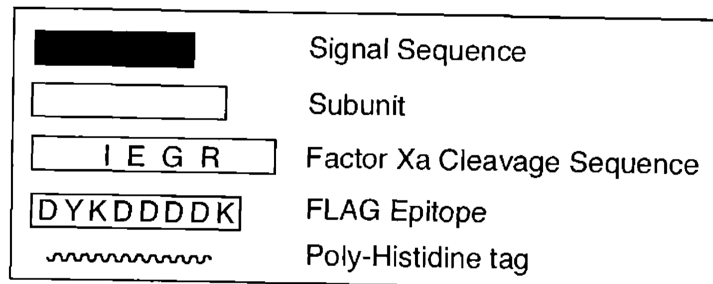


Figure II-2. Schematic of the four essential subunits of the yeast oligosaccharyl transferase.

Extraction of the largest fraction of proteins expressed in this system required the use of high concentrations of denaturing agents, such as 6 M guanidinium-HCl or 8 M urea. In order to limit the amount of manipulation required to assay for enzymatic activity, it was determined that the subunits should be purified from a protein expression system which did not require the use

of denaturing agents and therefore reconstitution of the *S. cerevisiae* oligosaccharyl transferase essential subunits expressed and purified from *P. pastoris* was abandoned.

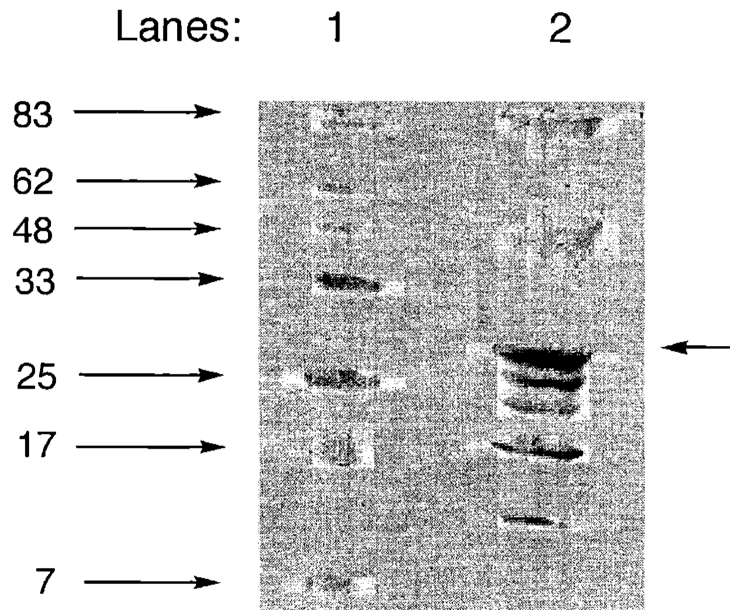


Figure II-3. Isolation of Swp1p, solubilized with 1% NP-40. Lane 1: molecular weight markers. Lane 2: Swp1p, identified using a polyclonal mouse antibody for this protein. The arrow on the right represents the expected molecular weight of Swp1p.

II-3 Expression of Native Subunits in *Spodoptera frugiperda* cells

The next objective was to express the proteins in the lytic baculovirus expression system. This system has proven successful in the functional reconstitution of a variety of membrane proteins, including several glycoproteins [14-17]. Nlt1p and Swp1p were cloned into the baculovirus vector pVL1393 since these proteins retained their native signal sequence. Wbp1p was cloned

into the pVT-BAC vector to utilize the signal sequence within this vector. Ost2p, which has an internal signal sequence was cloned into the pVT-BAC vector to ensure that this protein would be directed towards the secretory pathway. Each protein maintained the epitopes outlined in Figure II-2.

The vectors were transformed and expressed in *Sf9* cells using the lytic baculovirus expression system. For purification, the proteins were solubilized with 1.5% n-dodecyl-β-D-maltoside and isolated using Ni-NTA chromatography. Initial purification attempts included both manual and FPLC Ni-NTA columns. The manual column, which included incubating the resin with protein overnight, appeared to be more effective. Merely passing the protein solution through the resin, even at low flow rates (0.02 ml/min) did not allow tight binding of the protein to the resin. Previous protein purification from *S. cerevisiae* showed that the FLAG antibody enabled facile isolation of the complex, however the purity of the membrane proteins from *Sf9* cells could not be significantly improved using FLAG affinity resin [6]. Heparin chromatography could not be used as a general method for purification of these proteins, since only Nlt1p could be purified using this technique.

Several methods were attempted to concentrate the proteins in order to minimize the total volume of isolate for the reconstitution experiments. One such method involved running a second, smaller, Ni-NTA column or heparin column (for Nlt1p) in an attempt to elute the protein in a smaller volume. A second method that was attempted included surrounding dialysis bags which had been

filled with detergent-solubilized and purified proteins with high molecular weight polyethylene glycol (PEG). High molecular weight PEG absorbs the water from the protein sample generally resulting in increasing the protein concentration. However, although PEG concentrated the protein, it also concentrated the detergent up to 5-7% (since the detergent micelle cannot pass through the dialysis membrane), as determined by ^{31}P NMR, thus, this method of concentrating the desired protein was abandoned [18].

In order to isolate milligram levels of protein, four liters of baculovirus-infected insect cells were harvested 3-4 days post-infection, solubilized with 1.5% n-dodecyl-β-D-maltoside and loaded onto Ni-NTA resin in the presence of 15 mM imidazole. The column was washed with buffer containing 20 mM imidazole and eluted with buffer containing 500 mM imidazole. It was observed that protein elutions with linear gradients of imidazole resulted in a broad protein elution profile and that optimal elution of the desired protein was obtained with stepwise elution of the recombinant proteins at 500 mM imidazole.

Total protein yields were in excess of 1.5 mg per liter of culture. Estimates of purity were high as seen by Coomassie staining and Western blot analysis showed very low levels of protein degradation (Figure II-4). Western blot analysis for Nlt1p, Swp1p an Wbp1p were carried out with polyclonal mouse antibodies specific for the protein of interest, while Ost2p was identified with the use of a monoclonal antibody specific for the FLAG epitope. No Coomassie stain is shown for Ost2p since this protein does not stain well with this detection

method. The small mobility shifts between Coomassie Blue and Western blots in Figure II-4 of the same protein are due to variability in electrophoresis conditions and are not indicative of molecular weight differences.

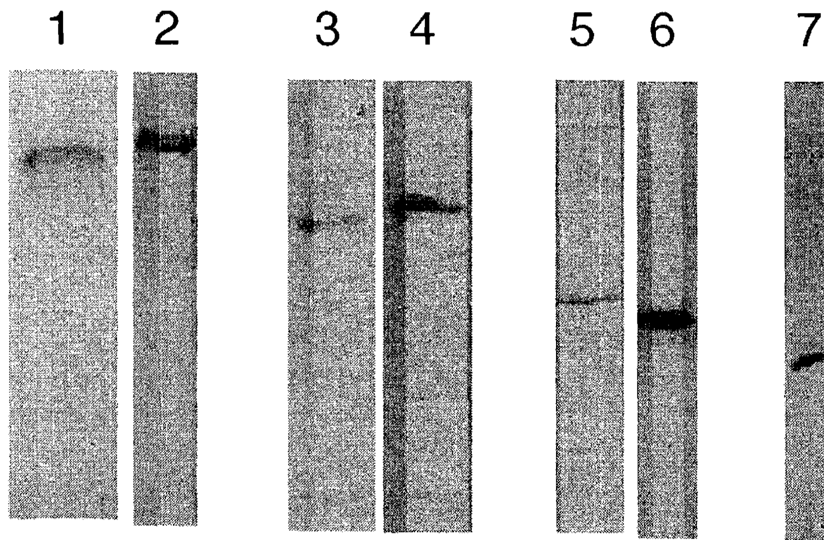


Figure II-4. Coomassie blue and Western blots of four essential subunits of oligosaccharyl transferase using 15% SDS-PAGE. 1., 3. and 5. are Coomassie Blue stains of Nlt1p, Wbp1p and Swp1p, respectively. 2., 4., 6. and 7. are Western blots of Nlt1p, Wbp1p, Swp1p and Ost2p, respectively. Each Western blot uses polyclonal antibodies specific for the respective protein, except for Ost2p which uses a monoclonal antibody specific for the FLAG epitope (Sigma Co.).

With the successful purification of the four essential subunits of the yeast oligosaccharyl transferase, the next experiments were focused on examining the oligosaccharide moieties on the two essential subunits which include potential N-linked glycosylation sites, Nlt1p and Wbp1p. N-linked glycosylation has previously been determined to be critical for the proper folding and presentation of glycoproteins [19]. In addition, different glycoforms have been shown to affect the thermostability and activity of RNAse B [20]. Due to the fact that insect cells

are capable of processing complex and hybrid N-linked oligosaccharides, it is important to determine the glycosylation state of these proteins as compared to oligosaccharides which are found on proteins expressed in yeast [17]. The primary sequences of Nlt1p and Wbp1p include four and two potential N-linked glycosylation sites, respectively. Therefore glycosylation of Nlt1p and Wbp1p was probed through the use of tunicamycin [21]. Tunicamycin inhibits the first step in oligosaccharide synthesis, the transfer of GlcNAc-P from UDP-GlcNAc to dolichol phosphate [22]. *Sf9* cells were infected with the appropriate virus and 1.0 μg/mL tunicamycin. The cells were harvested 72 hours after infection and the glycosylation states of Nlt1p and Wbp1p, the two essential and glycosylated proteins, were probed by western blot analysis as shown in Figure II-5. The glycoforms could only be separated using low acrylamide concentration during SDS-PAGE. These distinct bands were not observed in the previous SDS-PAGE due to the differences in acrylamide concentrations. Both proteins have two bands which collapse into a single band in the presence of tunicamycin. This suggests that both proteins are heterogeneously glycosylated in insect cells. Although neither protein is uniformly glycosylated, this should not be a significant issue for reconstitution experiments, since the proteins are not uniformly glycosylated in yeast [23].

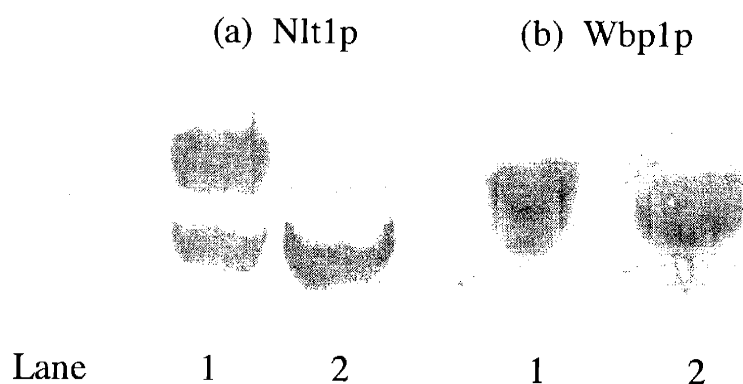


Figure II-5. Western blot analysis demonstrating the glycosylation state of recombinant Nlt1p (a) and Wbp1p (b) proteins expressed and purified in Sf9 cells using 8% SDS-PAGE. Lane 1: protein under native conditions. Lane 2: protein expressed in the presence of 1.0 μg/mL tunicamycin.

II-4 Assay Development

With the efficient expression and purification of four essential subunits from the enzyme, the next step was to improve the detection limits of the current OT assay. Previous assays utilized a tritiated disaccharide substrate, Dol-PPGlcNAc[^{3}H]GlcNAc [24, 25] (Figure II-6). In this assay, the reaction is quenched in an organic:aqueous mixture that partitions the tritiated glycopeptide product into the aqueous layer and separates the tritiated disaccharide substrate into the organic layer. The aqueous layer is subsequently quantitated by liquid scintillation counting.

Traditional organic-quenched assays, although effective in determining relative rates of reaction for inhibitor studies, are limited when the focus is on detecting low enzymatic activity due to the presence of tritiated disaccharide (GlcNAcGlcNAc) in the aqueous layer which has hydrolyzed from the dolichyl-diphospho-oligosaccharide substrate. This disaccharide resulted in a significant

background during the enzymatic assay. Therefore, a method was required that could differentiate low rates of reaction for this enzyme from hydrolysis of the dolicholpyrophosphate oligosaccharide substrate. The initial goal was to design a tight-binding substrate covalently attached to biotin that would enable use of streptavidin membranes for isolation of the glycopeptide.

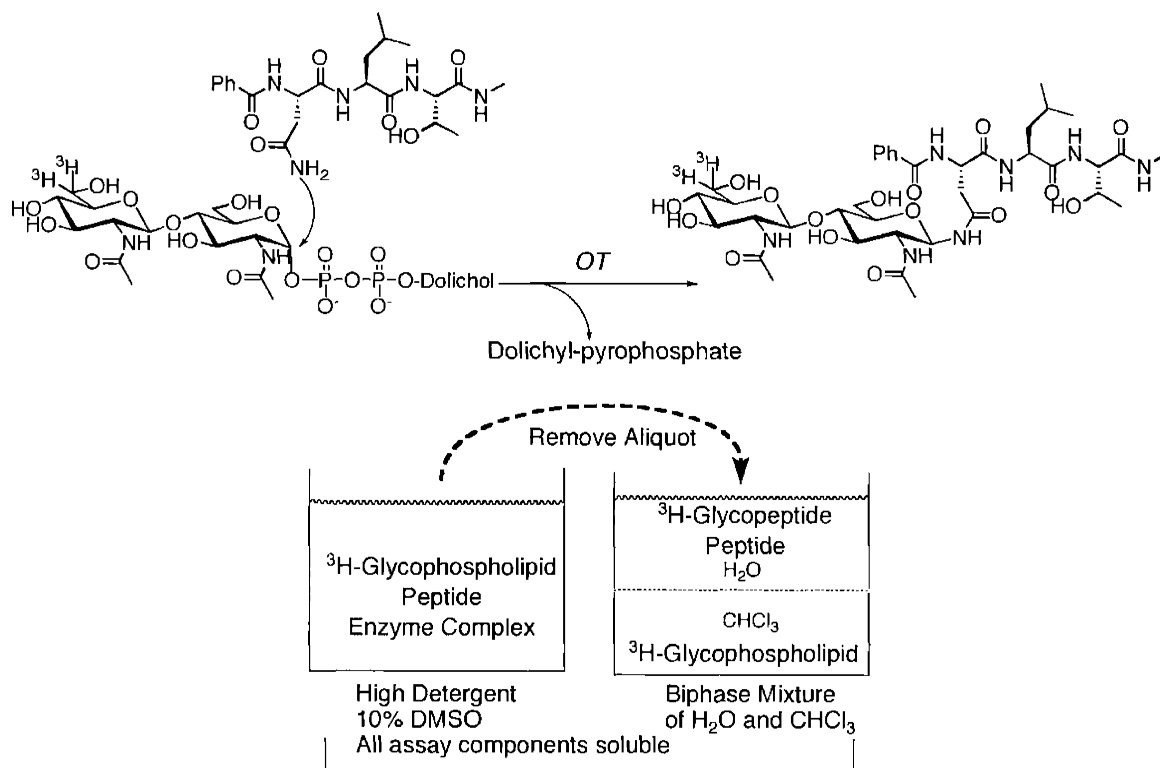


Figure II-6. Radiometric assay for oligosaccharyl transferase.

Solid-phase synthesis is a facile method for synthesizing and labeling polypeptides with biotin [26]. In order to probe the effect of using an extended substrate in a streptavidin-based assay system, a series of peptides was synthesized (Figure II-7). In **1**, the tripeptide is capped with a biotin label at the

N-terminus and a *p*-nitrophenylalanine at the C-terminus. The latter was later used to determine the peptide concentration. The tripeptide, BzNLTNHMe, has a K_m^{app} of 24 μM [27, 28]. Addition of the biotin moiety and *p*-nitrophenylalanine had no significant affect on the K_m^{app} of the substrate (24.5 μM). All K_m^{app} values were determined by plotting the data on a Hanes plot, where [S]/V is plotted as a function of [S], where [S] is the substrate concentration and V is the enzyme velocity. The X-intercept is equal to $-K_m^{app}$ (Figure II-8).

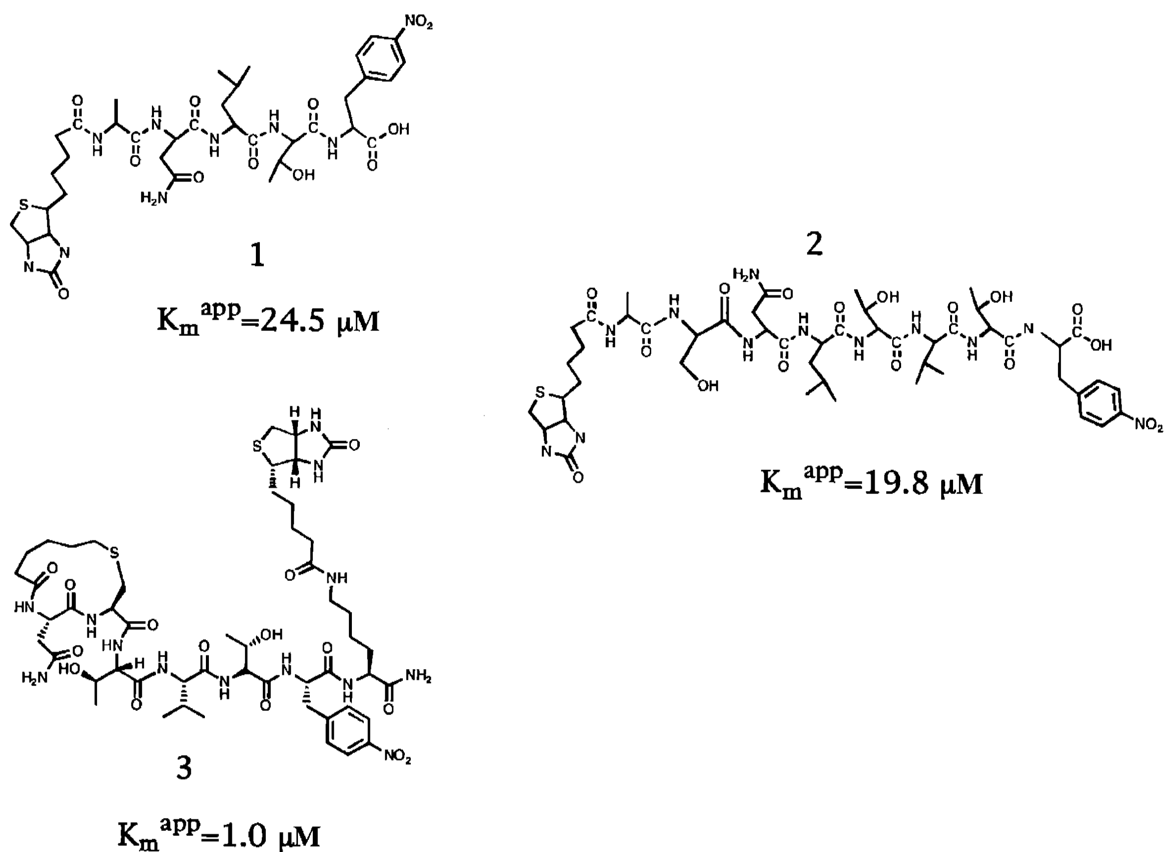


Figure II-7. Outline of Biotinylated Substrates for Oligosaccharyl Transferase.

It is necessary to use a tight-binding substrate in this assay because only 1.3 nmole of substrate can be bound to each streptavidin membrane (1 cm^2). In

order to prepare a more effective peptide substrate, an extended peptide substrate, **2**, was synthesized. Serine has previously been determined to be the optimal residue N-terminal to the asparagine of the consensus tripeptide [24]. The valine-threonine sequence located at the C-terminus of the tripeptide has previously been determined as the optimal sequence. The K_m^{app} for this substrate was 19.8 μM. Unfortunately, this modification did not appreciably improve the affinity of the substrate for the enzyme. A third peptide, **3**, was therefore synthesized which incorporated a cyclized domain to improve affinity by introduction of a constrained Asx-turn. Due to the loss of the N-terminus upon cyclization, biotin was added to a C-terminal lysine residue after deprotection and before cleavage from the solid-support. In this peptide, the apparent dissociation constant improved twenty-fold to 1 μM. This high affinity, small-molecule substrate was ideal for further experiments to examine the utility of a biotin-streptavidin system.

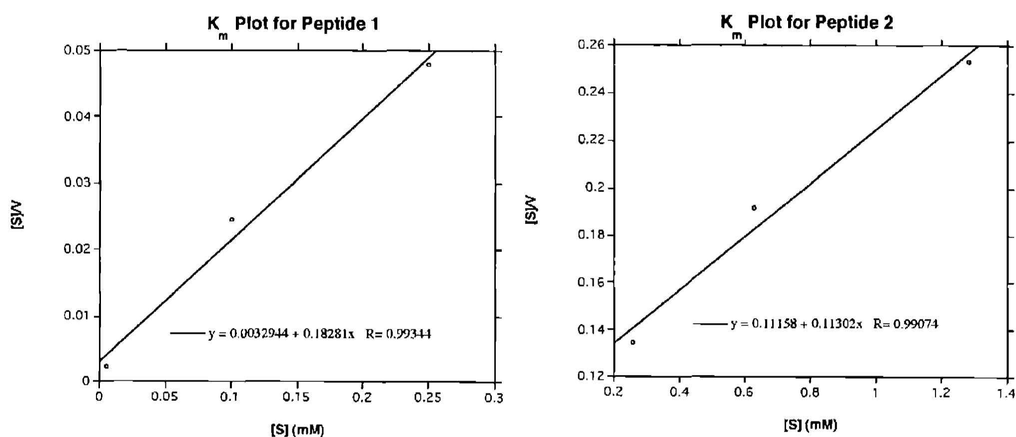


Figure II-8. Hanes plot to determine K_m^{app} of peptides 1 and **2**. The data for K_m^{app} of peptide 3 can be found in [18].

The assay mixtures were quenched into acidic stop buffers and subsequently loaded onto streptavidin-coated membranes. In order to have a sensitive assay, the dolicholpyrophosphate disaccharide substrate needed to be washed from the membrane. It was noted that dolichyl-PP-dissaccharide bound tightly to the membrane, presumably due to the hydrophobicity of the dolichyl substrate. Initial washes with sodium chloride resulted in high background counts, not significantly better than that seen for the organic-quenched assay protocol described previously. Subsequent washes with phosphoric acid and ethanol also failed to diminish the background counts of this assay. The addition of 1% Triton reduced the background counts twelve fold when compared to the organic-aqueous quenched assay (Figure II-9).

Only after these experiments were completed was a method described for covalent attachment of biotin to a tripeptide substrate by Coward and co-workers [29]. Unfortunately, the Y-intercept for the biotin-based assays was still too high to detect low levels of enzyme activity. Therefore, a more sensitive assay was required. However, this biotin-based procedure is superior to the organic quenched assay and is amenable to high-throughput screening for inhibitors of oligosaccharyl transferase.

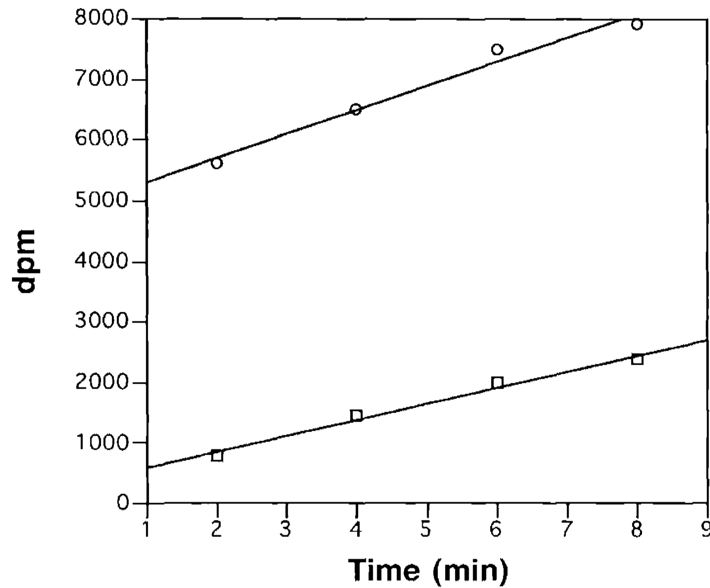


Figure II-9. A comparison of the hydrolyzed sugar substrates for the traditional organic quenched assay (open circles) and for the biotin-streptavidin based system (open boxes).

II-5 HPLC Assay

HPLC was used in an effort to obtain a highly sensitive enzymatic assay, which is able to quantitatively separate the dolichol-linked sugar substrate, hydrolyzed sugar substrate and glycopeptide product, thus facilitating mass spectrometry analysis. An example of the HPLC trace and scintillation counting values are shown in Figure II-10. In this plot, peptide and glycopeptide elution were measured using a linear gradient of 15-30% acetonitrile with 0.1% trifluoroacetic acid from 5 to 35 minutes on an analytical C_{18} column (microsorb

MV C18, 5μm, 100 Å, 25 cm) using a Beckman System Gold liquid chromatography system.

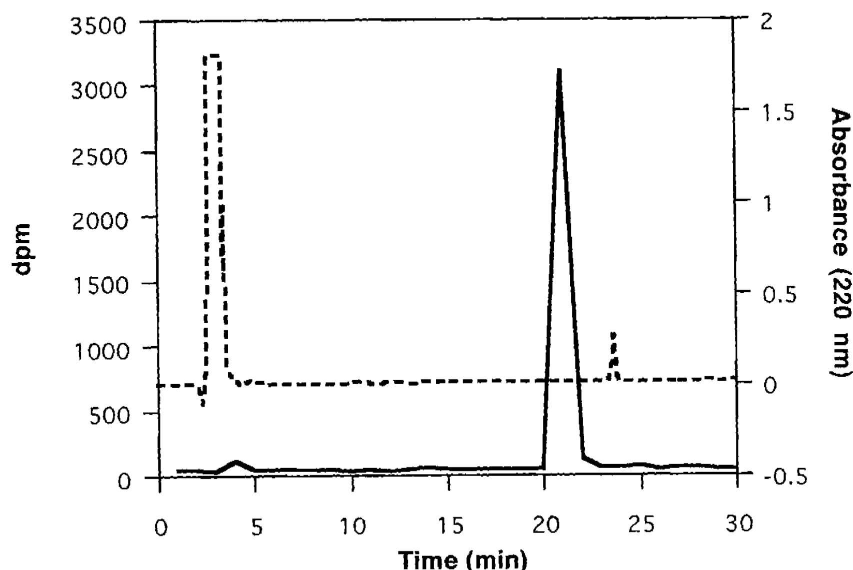


Figure II-10. An HPLC trace of oligosaccharyl transferase. Solid line represents radioactivity and broken line represents absorbance at 220 nm.

In this experiment, crude microsomes, partially purified from yeast were incubated for thirty minutes. The sample was quenched using the previously described organic:aqueous extraction method and the sample was loaded onto an C_{18} analytical HPLC column using a H_2O/acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. In this assay, the peak at 21 minutes (solid line trace) corresponds to the glycopeptide and the peak at 23 minutes (broken line trace) corresponds to the peptide substrate. The major peak at 3-5 minutes includes the DMSO and other buffer components that are eluted at the solvent front. The minor peak at the solvent front corresponds to hydrolyzed sugar

substrate. These data demonstrate that the hydrolyzed sugar substrate can be efficiently separated from the glycopeptide product after loading onto an analytical C_{18} column. In addition, the levels of detection for this assay are extremely low: Using a specific activity of 50 Ci/mmole of sugar substrate, a conservative detection limit of 200 dpm corresponds to 640 attomole of product. This detection level is significantly lower than the background counts seen for both the traditional organic:aqueous quenched assay and for the biotin/streptavidin assay system as previously shown in Figure II-9. In addition, the total recovery of radioactivity from the hydrolyzed disaccharide and glycopeptide after HPLC when compared to the sample before it was loaded onto the column is greater than 90%.

II-6 Electron Microscopy

With a sensitive assay for enzymatic activity, the next step in the project was to establish the liposome technology for membrane protein reconstitution. There are a number of methods one can use to reconstitute membrane proteins and these include the use of bicelles or heterologous expression systems such as *Xenopus ocytes* [30, 31]. Due to previous successes in the reconstitution of a variety of membrane proteins using liposomes, this system was chosen to investigate the reconstitution of oligosaccharyl transferase [32, 33]. In order to investigate the formation of liposomes during reconstitution, electron microscopy was used to follow liposome formation and the interaction of such with detergent-

protein micelles [34]. In summary, the proteins are purified in the presence of dodecyl β-D-maltoside which results in protein micelles. These micelles, depending on the size of the protein, can be in excess of 100 angstroms in diameter (Figure II-11) [35]. In these reconstitution experiments, liposomes were composed of phosphatidylcholine, and were preformed by sonication before addition to the detergent-solubilized proteins at a lipid to protein ratio of 6:1 (w:w). In order to remove the detergent and to form proteoliposomes, researchers have previously used dialysis or rapid dilution techniques to lower the concentration of detergent below the critical micelle concentration (CMC) [36]. Dialysis is an effective tool in reconstituting proteins that have been solubilized using detergents with high CMC's such as octylglucoside (21.8 mM) [34]. However, the CMC of dodecyl β-D-maltoside is 0.17 mM and therefore it is technically challenging to dilute this detergent sufficiently to form proteoliposomes [34]. An alternative approach is to use hydrophobic Bio-Beads to absorb detergent and therefore to decrease the concentration of dodecyl β-D-maltoside in this way.

As illustrated in Figure II-11, Bio-Beads, which are polystyrene beads with a high affinity for detergent, contain many small pores that selectively absorb detergent. Proteins and lipid micelles are excluded from these pores due to the larger size of these molecules. Thus, detergents can be efficiently and selectively removed from protein/lipid/detergent mixtures. In addition, the selection of detergent is critical to the orientation of proteins after the formation of

proteoliposomes. It has previously been established that the orientation of proteins within the proteoliposomes is dependent on the type of detergent used [37]. For instance, Triton X-100 has been shown to facilitate unidirectional incorporation of membrane proteins into preformed liposomes, while the use of dodecyl β-D-maltoside promotes a more random incorporation of membrane proteins [38, 39]. In addition, insertion of detergent-solubilized proteins into preformed liposomes has also resulted in proteins that have a more uniform directionality [40].

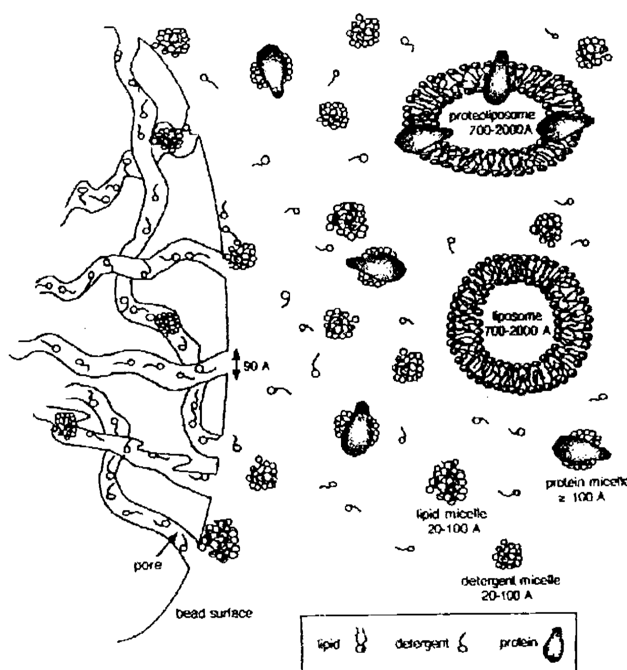
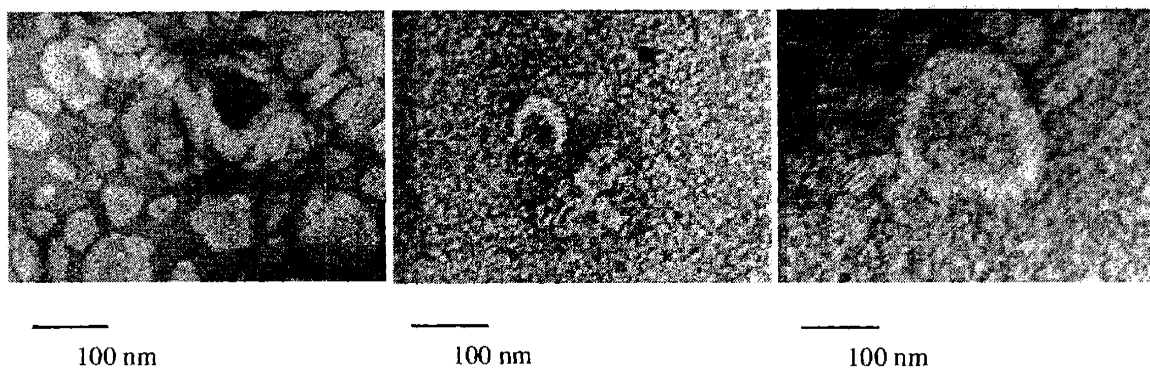


Figure II-11. Schematic of the use of Bio-Beads to facilitate the formation of proteoliposomes. Adapted from ref. [35].

In order to investigate the reformation of liposomes, electron microscopy was utilized (Figure II-12). In this experiment, liposomes, at concentrations

varying from 0.5-4.0 mg/ml, were examined in buffer. The panel on the left diagrams the formation of liposomes (at 2.0 mg/ml), before the addition of dodecyl β-D-maltoside, as circular structures at the center of the photo with dark interiors. After the addition of detergent, varying in concentrations from 1-1.5% w/v, no liposomes were visible (data not shown). Following the addition of Bio-Beads (24 hours), liposomes were identified in solutions originally containing 1 mg/ml phospholipid and 1% detergent (center panel). In addition, the formation of liposomes was also observed for a sample containing 4 mg/ml phospholipid and 1.5% detergent 24 hours after the addition of Bio-Beads. Thus, liposomes are able to reform after exposure to detergent followed by Bio-Beads. These data are consistent with ^{31}P NMR studies, which examined the presence of liposomes as a function of the tumbling time of the phospholipid head group in the presence and absence of detergent as performed by Dr. S. O'Connor [18].



100 nm　　100 nm　　100 nm

Figure II-12. Transmission electron microscopy of liposomes following the addition of detergent and Bio-Beads. The left panel shows liposomes formed after sonication. The middle panel displays liposomes formed after the addition of Bio-Beads to a solution containing 1 mg/ml phospholipid and 1% detergent. The right panel shows liposomes formed after the addition of Bio-Beads to a solution containing 4 mg/ml phospholipid and 1.5% detergent. Bio-Beads were incubated with the solutions for 24 hours.

Following this experiment, the next objective was to determine the appropriate detergent concentration for solubilizing liposomes by electron microscopy. The results of this experiment, performed at the Biomedical Facility at MIT, are shown in Figure II-13.

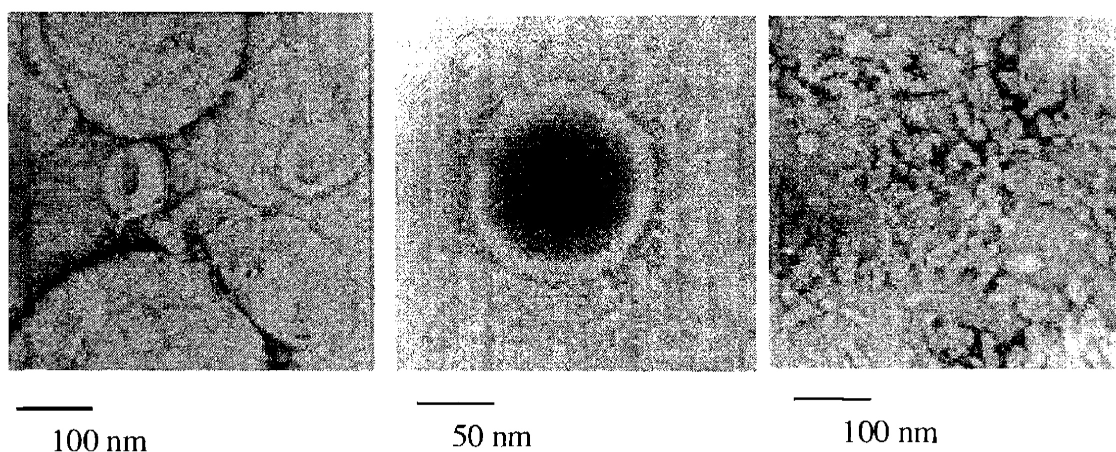


Figure II-13. Examination of the minimal amount of detergent required to disturb the stability of liposomes. The left panel shows liposomes at a lipid concentration of 2 mg/ml. The center panel contains liposomes with 2 mg/ml dodecyl β-D-maltoside. The right panel displays liposomes (2 mg/ml) with 4.0 mg/ml dodecyl β-D-maltoside.

As observed in the previous experiment, liposomes (2 mg/ml) are present in solution before the addition of detergent (panel on left). Addition of dodecyl β-D-maltoside up to a concentration of 2.0 mg/ml retains the presence of liposomes (center panel). Finally, the addition of detergent to a final concentration of 4.0 mg/ml results in the breakdown of liposomes, as demonstrated by the replacement of circular structures with linear lipid fragments (panel on right).

The electron microscopy experiments suggest that it was possible to disrupt preformed liposomes with detergent and then reform liposomes. With these results in hand, reconstitution of four essential subunits from oligosaccharyl transferase was initiated. Protein was expressed and purified from baculovirus-infected insect cells, incorporated into liposomes and tested for activity using the HPLC assay to determine whether the reconstituted sample was catalytically active.

II-7 Purification of Yeast Subunits from Endogenous Activity

The general outline of the first reconstitution efforts was to express each native yeast protein in *Sf9* cells, solubilize and purify the proteins using n-dodecyl-β-D-maltoside and a Ni-NTA column, respectively, incorporate the proteins into preformed liposomes and remove the detergent with Bio-Beads to form proteoliposomes. Over the course of these steps, the final volume of the proteoliposomes was greater than 10 ml. This large volume of sample was unwieldy to assay and a method was required to reduce the volume of the proteoliposomes.

Following the formation of proteoliposomes, the sample was diluted so that the sucrose, which is generally needed to stabilize the enzyme and at a concentration of 140 mM, is less than 50 mM. The sample was centrifuged for two hours at 50,000 rpm in the 70.1 ti rotor (Beckman Coulter Corp.) to pellet the proteoliposomes and the sample was resuspended in a small volume (60 μL) of

buffer for further analysis. Each subunit was detected from this sample by Western blot analysis, suggesting that the proteins were incorporated into liposomes after the removal of detergent.

It should be noted here that a second possibility exists for the presence of protein in the Western blot analysis without incorporation into the liposomes. This would be possible if the protein precipitated out of solution and was distinct from the liposomes. A sucrose gradient that suspends the proteoliposomes while pelleting any aggregated protein would distinguish between these two possibilities [41]. In these experiments, the proteoliposomes could be effectively isolated from aggregated protein and the presence of the protein could be verified by Western blot analysis, thus establishing the presence of protein in the proteoliposomes.

The next step was to assay activity for each of the individual subunits. These experiments are summarized in Figure 11-14. In this experiment, 100 μg of each protein was incorporated in liposomes and assayed for activity for 24 hours. Although the final molar concentrations of each protein is different due to the differences in molecular weights between proteins, these assays provide baseline data in regards to the activity of the individual subunits of the enzyme complex. Surprisingly, in this set of experiments, each individual subunit was active. This activity is time dependent and is proportional to the total amount of protein examined.

There are two possible reasons for the results of this experiment. The first possibility is that each subunit contains the ability to catalyze N-linked glycosylation, this is unlikely. A second hypothesis is that insect cell glycosylation machinery is being retained in the purification procedure. This is consistent with the fact that all previous purifications of this enzyme relied on affinity chromatography and association of the complex during purification for catalytic competency.

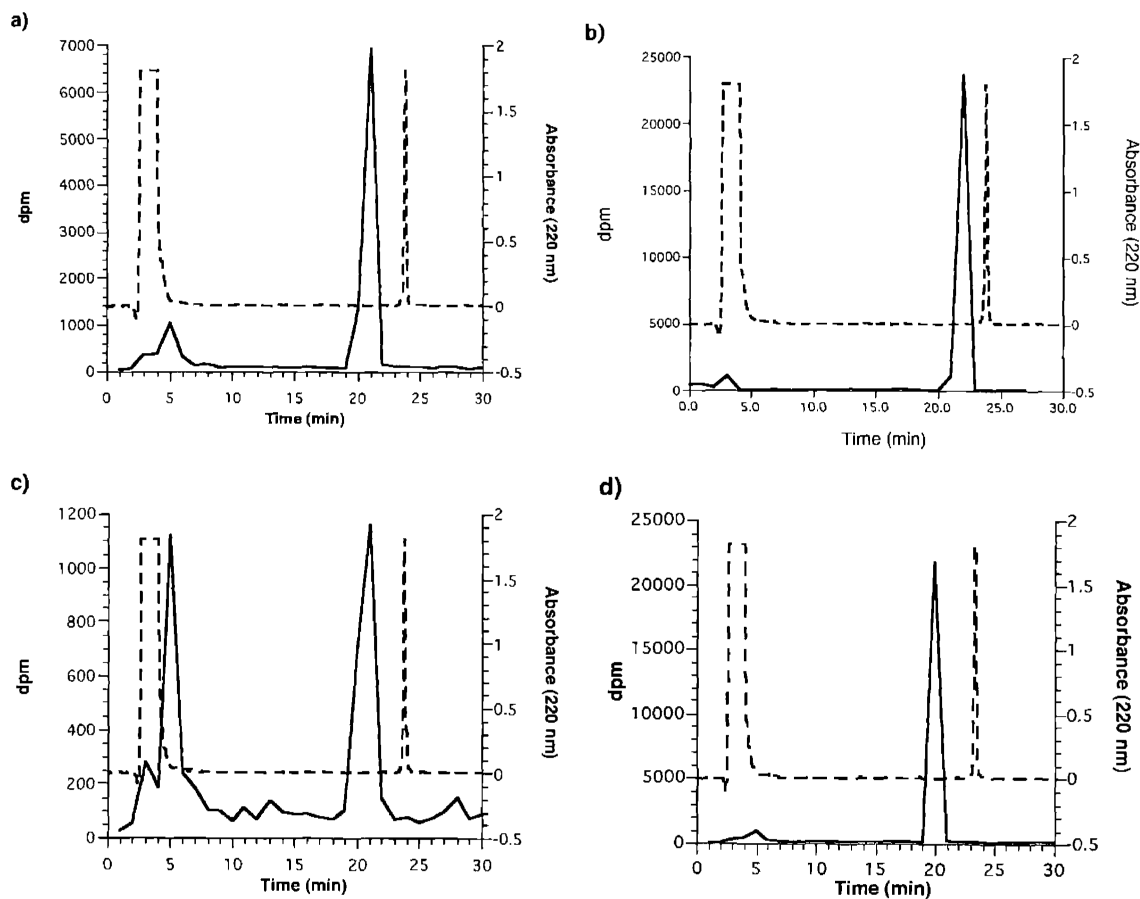


Figure II-14. Reconstitution of four essential subunits of oligosaccharyl transferase. For each plot, the solid line represents radioactivity and the broken line represents absorbance at 220 nm. The plots are as follows: a) Nlt1p, b) Ost2p, c) Swp1p and d) Wbp1p.

Two options were possible in order to eliminate the endogenous activity. The first is to utilize a species-specific inhibitor to N-linked glycosylation such as the use of ouabain in the studies on the Na^+/K^+-ATPase [42, 43]. Unfortunately, no species-specific inhibitor is available for this enzyme. A second method would be to physically separate the recombinant protein from the insect cell proteins. Since one would expect that any affinity purification step would retain these undesired proteins during purification, a more reasonable step would be to use methodology which could separate this enzyme complex from the desired recombinant protein. Thus, gel filtration chromatography was employed to purify and separate the yeast subunit from the yeast/insect cell complex. In this experiment, the protein, after Ni-NTA purification was loaded onto a gel filtration column. Following gel filtration chromatography and analysis by Western blot, two peaks were observed, one at the void volume of the column and one at a significantly lower molecular weight. For each protein, it was difficult to determine the exact molecular weight of the protein since it is part of a protein micelle and thus even in a monomeric mass would elute at a weight significantly larger than that determined by the calibration curve of the column.

II-8 Liposome Reconstitution

Each of the four proteins was expressed, purified using a three-step procedure (a final Ni-NTA column was utilized to increase protein concentration), incorporated into liposomes and assayed for activity. Examination of the

individual protein subunits, after the three-step purification, resulted in no detectable residual catalytic activity

The next objective was to mix the four proteins together, incorporate the subunits into liposomes and examine the proteins for activity. No activity was observed with this experiment (Figure II-15). One should note, however, the broad peak in the HPLC assay from 17-27 minutes. This peak is characteristic of all of the reconstitution experiments when performed in the presence of high concentrations of phosphatidylcholine. However, the total amount of radioactivity is extremely low and is not indicative of enzyme activity. In addition, in this experiment, Western blot analysis identified the presence of Nlt1p, Ost2p, Swp1p and Wbp1p, suggesting that the proteins were present but not active.

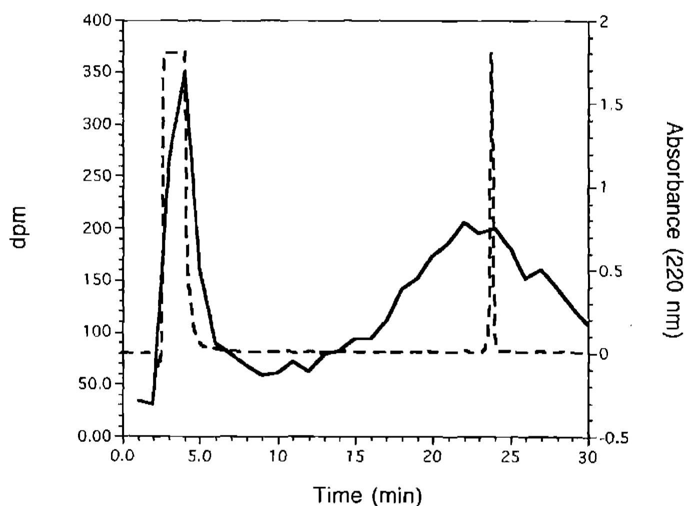


Figure II-15. Reconstitution assay of 100 μg each of Nlt1p, Ost2p, Swp1p and Wbp1p in liposomes; assay proceeded for over 24 hours. The solid line represents radioactivity and the broken line represents absorbance at 220 nm.

It is important to note that due to the gel filtration chromatography step, reconstitution experiments were limited since only 10% of the total protein was recovered after this step. Subsequently, multiple gel filtration chromatography experiments were required to obtain appreciable levels of protein. There were two working hypothesis as to why the yield after gel filtration chromatography was so low. The first is that the protein was sticking to the hydrophobic resin of the column and did not elute from the resin. The second hypothesis was that the protein, which eluted at the void volume of the column contained both the insect/yeast protein complex as well as aggregated recombinant protein and contained the majority of recombinant protein. In either case, it would be useful to alter the protein purification so that this chromatography step is not necessary.

II-9 Modified Protein Purification

To this end, both the purity and the concentration of the proteins were improved using an alternative methodology. The initial solubilization was still performed in n-dodecyl-β-D-maltoside, but all of the steps following solubilization were performed in Triton X-100 in the presence of low levels of dithiothreitol. Dithiothreitol is hypothesized to break up the association of the yeast/insect cell complex due to reduction of intermolecular disulfide bonds. An initial concern was that Triton X-100 or DTT might inactivate the enzyme, however it has been previously established that the enzyme is active in Triton X-100 and dithiothreitol

has been used to rescue the yeast enzyme after labeling with cysteine modifying reagents [44]. After the initial Ni-NTA column, the protein was assayed for activity. In these experiments, all four proteins were found to be inactive when assayed individually. In addition, combining these proteins after purification and then carrying out an enzymatic assay also resulted in no activity.

II-10 Assay of "Solubilized Wbp1p" and Wbp1 in Proteoliposomes

Previous work had been done under the assumption that a membrane environment was required for reconstitution of these transmembrane proteins in order to mimic their native environment. However, because the proteins examined in these experiments are not transporters, a functional membrane is not required for measuring the rate of reaction. In order to examine the importance of a functional membrane for catalysis, one fraction of Wbp1p, purified in a one-step protocol from insect cells and therefore, retaining catalytic activity from the endogenous insect cell oligosaccharyl transferase, was examined both in liposomes and in solution. It had been previously known that protein purified in a one-step protocol were active in liposomes, and this activity had previously been attributed to proteins from the insect cells. Surprisingly both assays appeared to be active, suggesting that the proteins need not be in a lipid bilayer for catalysis (Figure II-16).

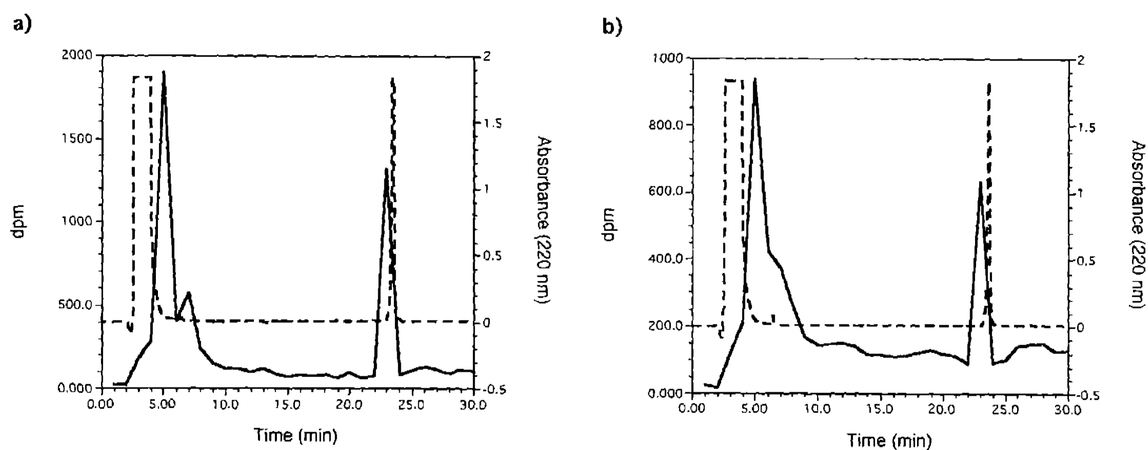


Figure II-16. A comparison of the activity of Wbp1p attributed to the yeast/insect cell complex in solution and in liposomes. In both plots, the solid line displays the radioactivity, while the dotted line represents the absorbance at 220 nm. Part a) shows Wbp1p in liposomes and part b) shows Wbp1p in detergent solubilized micelles.

II-11 Reconstitution in Solution

The next step was to examine the activity of the protein complex in solution as expressed in insect cells (Figure II-17). No broad peak is visible in Figure II-17 as was present on previous reconstitution experiments, due to the absence of a high concentration of phosphatidylcholine. This is an advantage since the lower background could facilitate identification of lower levels of enzymatic activity.

In these experiments, no activity was observed for each subunit after purification from baculovirus-infected insect cells. In addition, reconstitution of the subunits in each possible set of two, three and all four subunits did not result in any detectable levels of activity. These data suggested that the proteins were

either not sufficient for catalysis or that the proteins were not in the correct orientation in the reconstitution experiment.

In order to optimize the orientation of these proteins, the next experiment examined whether the proteins could be co-expressed using the baculovirus-infected insect cell system, co-purified and then examined for activity (Figure II-15). In this assay, no activity was observed even when the proteins were co-purified in the presence of Triton X-100 and DTT. These data suggest that something is absent in these reconstitution studies which was critical for enzymatic activity.

To further expand the number of proteins in the reconstitution experiments, the last essential subunit, Stt3p, from the yeast oligosaccharyl transferase was studied. This protein was initially believed to be a sub-stoichiometric subunit involved in protein organization and was thus not initially included in reconstitution experiments [45, 46]. In the following experiments, a clone of Stt3p fused to Protein A for facile purification and expressed in yeast was obtained from M. Aebi (ETH, Zurich, Switzerland) in order to ascertain the importance of this protein in enzymatic activity. This protein was originally believed to be a substoichiometric factor in the assembly of the yeast oligosaccharyl transferase. In addition, the protein has been difficult to identify since the Stt3p has not been amenable to facile antibody production, does not stain with Coomassie Blue or Silver stain and the mobility of the protein on SDS-PAGE is significantly smaller than the predicted molecular weight mass.

However, within the past year, research has identified that this protein is critical to the function of the enzyme [3, 47, 48].

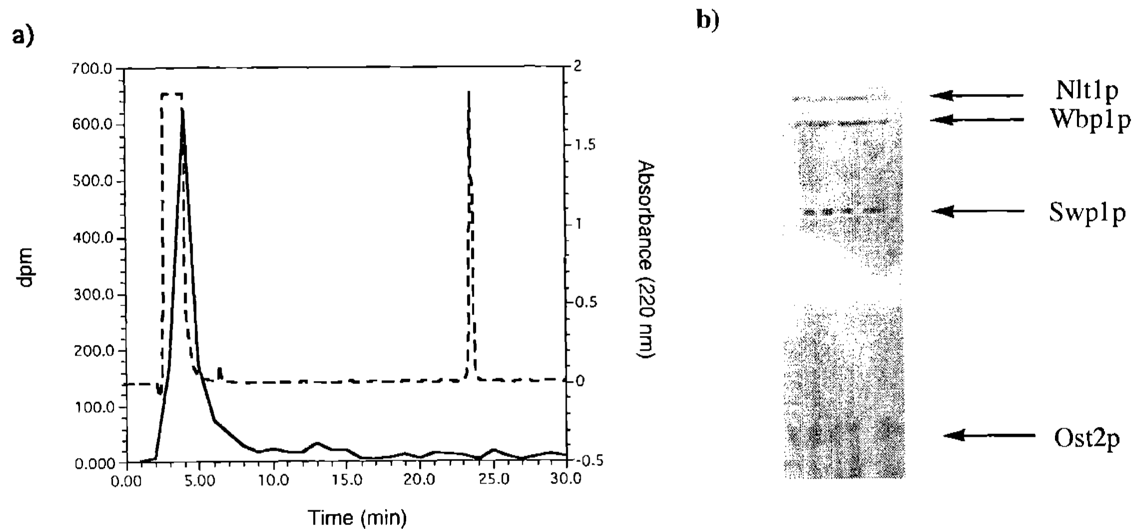


Figure II-17. Reconstitution of co-expressed and co-purified Nlt1p, Ost2p, Swp1p and Wbp1p. Part a) displays the results of the HPLC assay with the solid line representing radioactivity and the dotted line representing absorbance at 220 nm. In part b), a western blot using the monoclonal antibody specific for the FLAG epitope identified the four essential subunits purified using Ni-NTA.

II-12 Stt3p Reconstitution Experiments

In order to probe the importance of Stt3p in oligosaccharyl transferase, a clone of *S. cerevisiae*, which contains a Stt3p-Protein A conjugate was obtained from M. Aebi. This clone was expressed in *S. cerevisiae* and was purified from yeast [49]. The protein was purified according to previously described methods, verified with a goat anti-rabbit antibody and visualized with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP) (Figure II-18) [49].

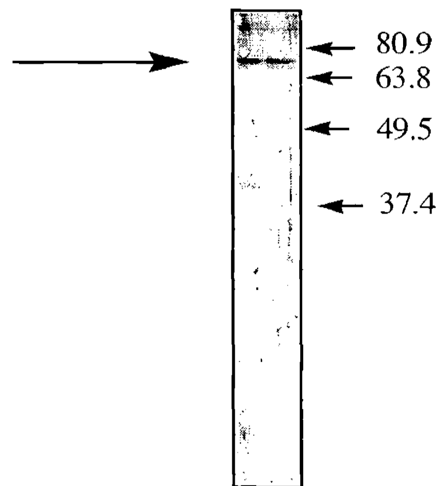


Figure II-18. Western blot of Stt3p-Protein A conjugate purified from *S. cerevisiae* including molecular weight standards.

Nlt1p, Ost2p, Swp1p and Wbp1p were concurrently purified from Sf9 cells. During these experiments, it was observed that elution of the recombinant proteins using imidazole gradients resulted in the purification of different populations of recombinant proteins at different imidazole concentrations. The hypothesis was that it took higher concentration of imidazole to elute protein which was associating with each other since the complex would contain multiple poly-histidine tags in close proximity. In order to optimize isolation of protein which was associating, the proteins were eluted with 500 mM imidazole. The protein was visualized using a FLAG antibody, followed by a secondary rabbit anti-mouse antibody-alkaline phosphatase conjugate with NBT/BCIP (Figure II-19).

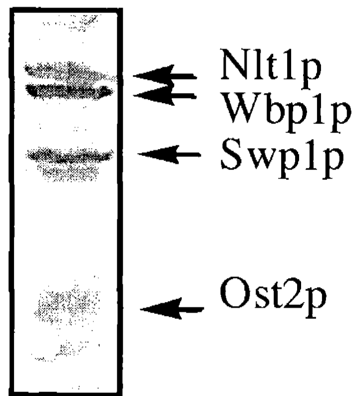


Figure II-19. Co-expression of Nlt1p, Ost2p, Swp1p and Wbp1p purified from Sf9 cells and visualized by western blot using the monoclonal antibody specific for the FLAG epitope.

In order to determine if the proteins were catalytically active, the HPLC assay was employed and the total conversion to glycopeptide was measured by radioactivity. Two experiments were initiated: a 30 minute assay and an end-point assay, where the assay proceeded for 24 hours before quenching. These two time points were chosen to examine the activity of the complexes during a more "kinetic"-like time point and to determine the total amount of turnover of the enzyme, respectively. In the 30 minute assay, the Stt3p-Protein A fusion protein had a significant amount of activity, which increased as a function of concentration (Figure II-20). The assay of the tetramer from insect cells did not yield appreciable activity after thirty minutes. When the two sets of protein were combined, there was no appreciable increase in activity from the combination of proteins when compared to the equivalent amount of Stt3p assayed individually. This result suggests that the tetramer of proteins is not sufficient for catalysis, but that Stt3p is required for a functional enzyme.

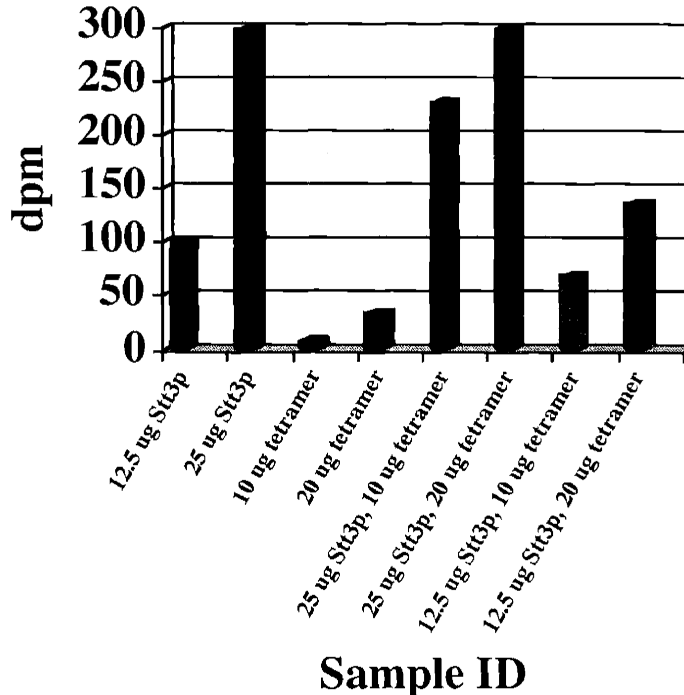


Figure II-20. A 30 minute assay of variable concentrations of the Stt3p-Protein A clone and the proteins co-expressed in insect cells.

Genetic studies of the enzyme complex have shown that there are five essential subunits for the enzyme. Deletion of any of these five proteins results in a lethal phenotype. Deletion of the remaining four nonessential subunits results in viable cells with a phenotype of decreased cell growth and/or a reduced efficiency of glycosylation. Reconstitution of four of the essential subunits purified from baculovirus-infected insect cells suggest that the Nlt1p/Ost2p/Swp1p/Wbp1p combination is not sufficient for catalysis or that the four proteins are not properly reconstituted for activity. These results taken with the activity shown with Stt3p suggest that this protein is required for catalytic competency. These data can not differentiate between whether Stt3p is

sufficient for catalysis or if one or more of the essential subunits are being retained during purification and if these additional essential subunits are required for catalysis.

The second assay examined the relative rate of activity after 24 hours (Figure II-21). As was seen with the earlier experiment, when the total amount of Stt3p is doubled from 12.5 to 25 μg, the amount of glycopeptide product doubles (1189 to 2340 dpm), while 20 μg of tetramer resulted in only 5 dpm, which can be considered background. Combining these proteins did not result in an appreciable change in activity when compared with Stt3p on its own.

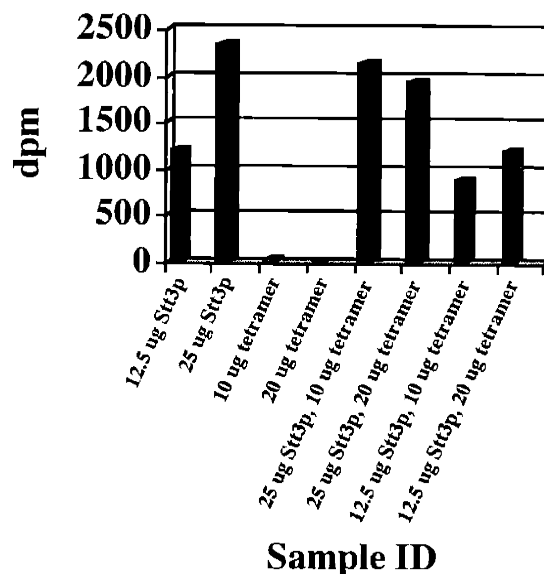


Figure II-21. A comparison of the relative total activity of the Stt3p-Protein A conjugate and the tetramer of proteins expressed and purified from Sf9 cells.

These data complement the data shown above in which Stt3p appears to be the primary component of activity. Although this protein could be retaining other components from the complex during purification, we can determine from previous experiments that the Nlt1p/Ost2p/Swp1p/Wbp1p complex, as currently reconstituted, is not sufficient for catalysis, but requires Stt3p for a functional enzyme. However, it cannot be definitively determined from these data whether Stt3p by itself is sufficient for catalysis. One method, in an attempt to isolate a pure Stt3p would be to purify the protein as previously described, unfold the protein in the presence of reducing and denaturing agents, purify the protein using gel filtration chromatography, refold the protein and assay for enzymatic activity.

The above experiment only describes the activity of the subunits of the enzyme and does not attempt to determine whether the proteins are interacting with each other under the assay conditions. Thus, two possibilities exist as to the relative state of the proteins in these reconstitution experiments (Figure II-22). In the first state, both the Stt3p-Protein A conjugate and the tetramer of proteins are interacting with each other in solution, while in the second state, the proteins are acting as two distinct entities and are not interacting with each other. In order to test the interaction of the two sets of proteins under the assay conditions, the proteins were incubated together, and bound to a IgG-Sepharose column, which should adsorb the Stt3p-Protein conjugate. If the tetramer of proteins is interacting with Stt3p, we would expect these proteins to elute from the column

when Stt3p is eluted from this affinity column. If, on the other hand, there are no interactions between these proteins, the tetramer would wash through the column independently of Stt3p.

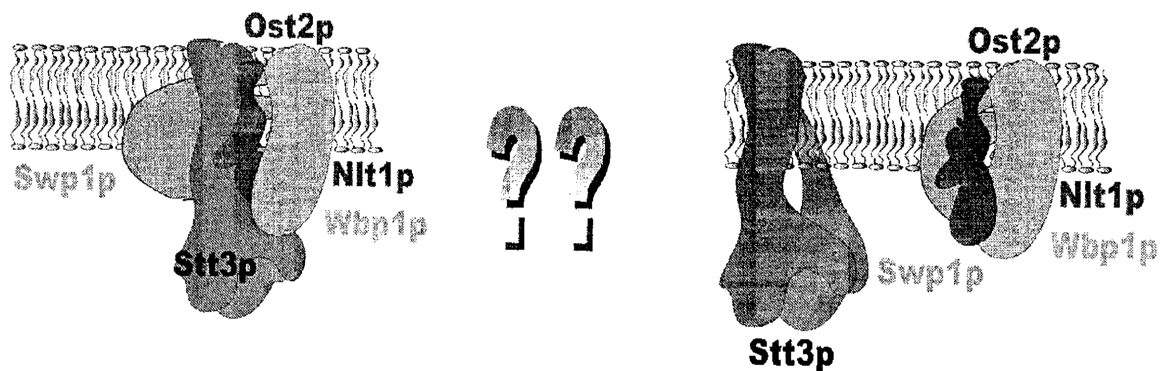


Figure II-22. Two possible states of the reconstitution experiment with the Stt3p-Protein A conjugate and the tetramer of proteins expressed in Sf9 cells. On the left is a diagram of the proteins interacting with each other, while on the right is a diagram depicting Stt3p independent from the tetramer of proteins in insect cells.

In order to verify whether these proteins are interacting with each other in solution, an antibody-based "pull-down" experiment was initiated where both proteins were combined and loaded onto IgG Sepharose. The proteins were washed and eluted to look for the presence of Stt3p and the tetramer. Figure II-23 demonstrates the results of this experiment. In the second lane, the proteins are combined and loaded onto the IgG-Sepharose column. Lane three shows that some of the heterologously expressed protein is washed away in the flow though, while no appreciable protein is lost upon washing the column at pH 5.0.

When the column is washed with buffer at a pH of 3.5, both the Stt3p conjugate and the insect cell proteins are eluted concurrently, suggesting that the proteins are interacting with each other under assay conditions.

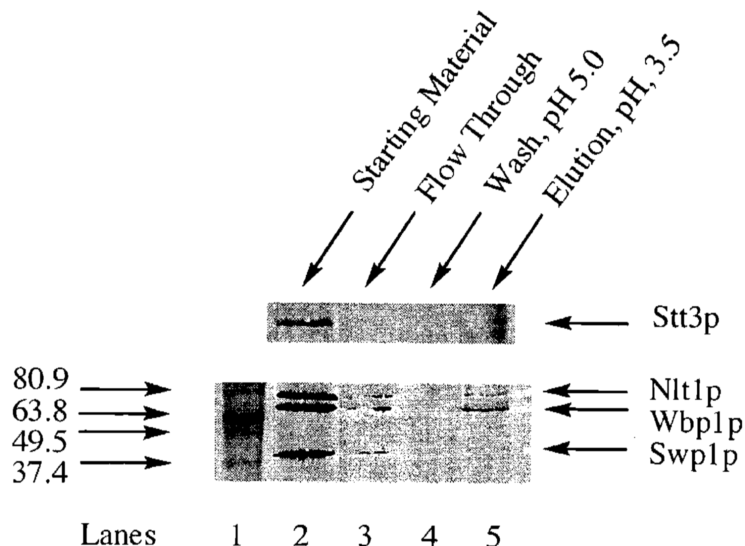


Figure II-23. A pulldown experiment with Stt3p and Nlt1p/Swp1p/Wbp1p. In this experiment, the first lane contains protein molecular weight markers, the second lane contains the starting material before loading onto the IgG column, the third lane contains the sample that flowed through the column upon loading the sample, the fourth lane demonstrates the protein eluted under mild pH conditions, while the fifth lane contains protein which was eluted from the IgG Sepharose column after the addition of strong acid. Stt3p is visualized using a secondary goat anti-rabbit antibody conjugated to alkaline phosphatase and the three proteins, Nlt1p, Swp1p and Wbp1p were visualized using a monoclonal antibody specific for the FLAG epitope. The antibody was not sensitive enough to detect Ost2p in any of the samples.

Combined with the previous assay data, these data suggest that the four proteins, Nlt1p, Ost2p, Swp1p and Wbp1p are not sufficient for catalytic activity but they are interacting with the Stt3p-Protein A conjugate. Instead, Stt3p, which

was originally identified as a substoichiometric protein involved in the assembly of this enzyme, is required for catalysis.

II-13 Discussion

Oligosaccharyl transferase catalyzes the first step in N-linked glycosylation. All previous purification attempts for this enzyme have included affinity chromatography from the native source, *S. cerevisiae*. These purification protocols are insufficient, as only nonlethal mutations of the enzyme can be used to probe the mechanism of catalysis. The purpose of the work presented in this chapter was to express the essential proteins in a heterologous system, purify them and reconstitute enzymatic activity. Previous purifications of the enzyme suggested that Nlt1p, Ost2p, Swp1p and Wbp1p were sufficient for catalysis. To this end, four essential subunits of the oligosaccharyl transferase were cloned and expressed in *P. pastoris*. The proteins in this expression system were highly aggregated and prone to degradation. Subsequent expression in the lytic baculovirus expression system provided protein that was readily solubilized in n-dodecyl-β-D-maltoside and could be purified using Ni-NTA chromatography. A new assay was developed, taking advantage of the strong association of biotin to streptavidin, and a modification of the HPLC assay was made enabling detection of attomole levels of activity for the enzyme. Electron microscopy was used to follow the formation, disruption and reformation of liposomes using Bio-Beads.

Initial attempts at reconstitution of enzymatic activity proved unsuccessful due to high levels of background activity from the insect cell expression system. Subsequent purification steps including gel filtration and heparin chromatography, as well as the use of reducing agents and alternative detergents decreased the level of endogenous activity to a negligible level.

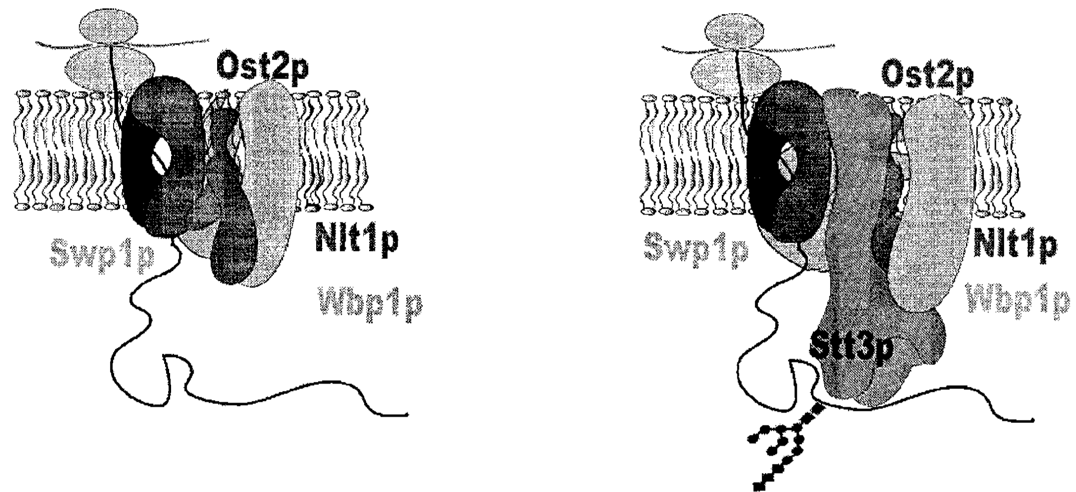


Figure II-24. Original model of essential subunits of the yeast oligosaccharyl transferase required for catalysis (left) and new working model of proteins sufficient for catalysis (right).

Expression of these proteins and reconstitution either into lipid membranes or as a detergent-solubilized species did not yield a functional

enzyme. Further reconstitution studies of Stt3p, with and without the four other essential subunits suggested that this protein does interact with the tetramer of proteins and is involved in enzymatic catalysis. Stt3p, which was originally identified as a substoichiometric factor, now appears to be critical for catalysis (Figure II-24). In order to definitively determine the role of Stt3p in catalysis, current efforts in the lab are focused on expressing Stt3p in insect cells as well as inactive mutants of this protein [48].

II-14 Acknowledgements

Thanks to P. Snow, Ph.D. at the Protein Expression Facility at the California Institute of Technology for introduction of the lytic/baculovirus expression system. In addition, Dr. S. O'Connor synthesized and characterized the cyclized biotin substrate.

II-15 Experimental Section

Expression of Native Subunits in P. pastoris. Subunits were cloned into pPicZ vectors (Invitrogen Inc., by C. Davis, R. Pathak and T. Walkup) and transformed into *P. pastoris*. Transformation included isolating a freshly streaked yeast cell line, SMD1168 (Invitrogen), from a frozen stock and growing this in culture (50 ml) to an optical density of approximately 0.8 at 600 nm. The cells were centrifuged at 8K rpm for 10 min at room temperature and washed twice with sterile water. The cells were resuspended in 1 ml of 100 mM $LiCl_2$ and

centrifuged for ten min at 14K rpm. The pellet was resuspended in 400 μL of 100 mM $LiCl_2$ and 50 μL aliquots were removed from this sample for each transformation. The cells were transformed by pelleting the cells and adding 240 μL 50% PEG-3350, 36 μL 1 M $LiCl_2$, 25 μL 2 mg/ml salmon sperm DNA, and 50 μL linearized plasmid DNA. The sample was incubated at 30 C for 30 min, heat shocked for 20 min at 42 C and the cells were pelleted. The cells were resuspended in 1 ml of nonselective media for one h and plated onto Zeocin-selective plates. A freshly streaked colony, plated on YPD (10 g BactoYeast extract, 20 g BactoPeptone extract and 20 g dextrose in one liter H_20) plus Zeocin (Invitrogen), was inoculated into 25 ml MGYH media [100 ml 10X yeast nitrogen base (34 g yeast nitrogen base and 100 g ammonium sulfate in 1 liter H_2O), 2 ml 500X Biotin (20 mg biotin in 100 ml H_2O), 10 ml glycerol and 10 ml 100X histidine (400 mg L-histidine in 100 ml H_2O)] for 24 h at 30 C. This culture was inoculated into 1 L of MGYH overnight. The A_{600} nm, was determined and eight, 800 ml aliquots of MMH media (80 ml 10X yeast nitrogen base, 1.6 ml 500X biotin, 8 ml methanol and 8 ml 100X histidine) were inoculated to an OD of 0.75. The culture was incubated at 30 C for 48 h and 100 ml of methanol was added after 24 h. The cells were centrifuged (8K rpm, 10 min, 4 C) and resuspended in TMI [50 mM Tris-Cl, pH 7.5, 2.5 mM $MgCl_2$, 0.1 mM AEBSF (4(2-Aminoethyl)benzenesulfonyl fluoride), 0.5 μg/ml Pepstatin A, and 0.5 μg/ml Leupeptin]. The cells were broken open with a bead beater (30 s on, 30 s off, 8 cycles) and the product was centrifuged (8K rpm, 10 min, 4 C). The supernatant

was saved for analysis. The pellet was resuspended in TMI and centrifuged (8K rpm, 10 min, 4 C). The supernatants were combined and centrifuged (40K rpm, 60 min, 4 C). The supernatant was saved and the pellet resuspended in RMI (140 mM sucrose, 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 0.1 mM AEBSF, 0.5 μg/ml Pepstatin A, and 0.5 μg/mL Leupeptin) plus 500 mM NaCl. After shaking on ice for 15 min, the sample was centrifuged (50K, 20 min, 4 C). The supernatant was saved, the pellet resuspended in RMI plus 0.05% NP-40, shaken on ice for 15 min, and centrifuged (50K rpm, 20 min, 4 C). The supernatant was saved, the pellet resuspended in RMI plus 500 mM NaCl and 0.6% NP-40, shaken on ice for 15 min, and centrifuged (50K rpm, 20 min, 4 C). The supernatant was saved and the pellet was resuspended in RMI plus 2M guanidine, shaken on ice for 15 min and centrifuged (50 K rpm, 20 min, 4 C). The supernatant was saved, the pellet was resuspended in RMI plus 4M guanidine, shaken on ice for 15 min and centrifuged (50K rpm, 20 min, 4 C). The supernatant was saved, the pellet was resuspended in RMI plus 6 M guanidine-HCl, shaken on ice for 15 min and centrifuged (50K rpm, 20 min, 4 C). The supernatant was saved and the pellet resuspended in RMI. Samples were run on a 10% SDS-PAGE.

Expression of Native Subunits in Spodoptera frugiperda. Nlt1p, Ost2p, Swp1p and Wbp1p were cloned into the lytic baculovirus/insect cell expression system using standard PCR techniques [50, 51]. Initial cloning of the genes from *S.*

cerevisiae was performed by C. Davis, Ph.D. and R. Pathak, Ph.D. Incorporation of the genes into baculovirus viral vectors was completed according to the methods of O'Reilly et al [52]. The expression vector pVL1393 from Invitrogen was used for Nlt1p and Swp1p since these proteins contained their native signal sequence while the pVT-Bac vector was used for Ost2p and Wbp1p since a signal sequence needed to be added to the N-terminus of this protein to ensure proper processing the protein. Recombinant virus was generated by cotransfection of the transfer vector the linearized viral DNA, Baculogold. All cells were grown in Sf 900 media (Gibco) supplemented with 10% fetal bovine serum (JRH Biosciences) and 0.5X Pen/Strep (Gibco). Cells were grown in one liter batches in fernbach flasks at 27 C on a rotary shaker at 100 rpm. Recombinant virus was amplified and titered using Sf9 cells with a multiple of infection (M.O.I) of 0.3. Protein expression was initiated using cells at a density of $3x10^6$ cells/ml and a M.O.I. of 5. Cells were harvested three to four days post-infection when cell viability was 60-80%.

Traditional Radiometric Assay. The assay was performed according to Imperiali and Shannon and based on the protocol by Sharma et al [53, 54]. Briefly, 30,000 dpm [^{3}H]-dolichylpyrophosphoryl-*N,N'*-diacetylchitobiose (specific activity 9 or 50 Ci/mmole) was dried down in an Eppendorf tube. Crude microsomes, partially purified from yeast and assay buffer (50 mM Tris-HCl, pH 7.5, 1.2% Triton X-100 and 10 mM $MnCl_2$) were added to each tube. The tri-peptide (Bz-NLT-NHMe), in

10 μL DMSO, was used to initiated the reaction. The reaction was quenched by the removal of 40 μL of the assay solution after 2, 4, 6 and 8 min into 1.2 ml 3:2:1 chloroform:methanol:4 mM $MgCl_2$. The aqueous phase containing the glycopeptide product was quantified by scintillation counting using Ecolite (ICN).

Peptide Synthesis. Amino acids and resins were purchased from PerSeptive Biosystems and Bachem, U.S.A. SAM^2 biotin capture membranes were purchased from Promega Corp. (Madison, WI). All other reagents were purchased from Sigma Chemical Co. and Aldrich Chemical Co. Linear peptides were synthesized using standard Fmoc (9-fluorenylmethoxycarbonyl) solid phase synthesis protocols from Fmoc PAL PEG resin (0.4 g, 0.17 mmol/g) on a Milligen 9050 PepSynthesizer [55]. Amino acids were coupled through the use of preactivated pentafluorophenyl esters or through the use of diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt). To couple biotin to the N-terminus of peptides following peptide synthesis, NHS-Biotin was activated with HOBt in DMF for two h with bubbling nitrogen. Upon completion of the linear peptide synthesis, the amino terminus was capped with a large excess of 6-bromohexanoic acid anhydride. Cyclization occurred between the thiolate of cysteine and the 6-bromohexanoyl group in degassed DMF using a large excess of 1,1,3,3-tetramethylguanidine as a base for 24 h [56]. Peptides were cleaved from the resin in a trifluoroacetic acid (TFA)/water/triisopropylsilane (TIPS) (95/5/5) mixture for three h, triturated in diethyl ether (2X), redissolved in

water/acetonitrile and lyophilized to dryness. All peptides were purified by preparative reverse phase high-pressure liquid chromatography (RP-HPLC) with a gradient elution (15-20% acetonitrile/water/0.1% TFA to 40-45% acetonitrile/water/0.1% TFA) over the course of 35 min, with a flow rate of 10 ml/min for a preparative C_{18} column. Peptides were verified using electrospray mass spectrometry. Peptide concentrations were determined by A_{280} of *p*-nitrophenylalanine [57].

Oligosaccharyl Transferase Assays. A solubilized membrane of yeast (*Saccharomyces cerevisiae*) OT was prepared as described previously [44]. Briefly, 150 g of RPY40 yeast cells were resuspended in 150 ml TM (50 mM Tris-Cl, pH 7.5 and 2.5 mM $MgCl_2$) and centrifuged in type 16 rotor (7,000 rpm for 8 min at 4 C). The cells were washed twice more with TM as described above. The cells were resuspended in TMI (TM plus 0.1 mM AEBSF, 0.5 μg/mL pepstatin A and 0.5 μg/mL). The cells were lysed in a bead beater for 16x20 s interval with 40 s on ice in between. The cell lysate was centrifuged in type 16 rotor (8,000 rpm for 10 min at 4 C). Following this step, the microsomes were pelleted from the supernatant by centrifugation in a type 45ti rotor (40,000 rpm for 60 min at 4 C). The pellet was resuspended in 75 ml RMI (50 mM Hepes, pH, 7.5, 140 mM sucrose, 2.5 mM $MgCl_2$ 0.1 mM AEBSF, 0.5 μg/mL pepstatin A and 0.5 μg/mL) and homogenized using a ground glass hand homogenizer. NaCl was added to the sample to 0.5 M and shaken on ice for 15 min. The salt-

washed microsomes were centrifuged in a type 45ti rotor (40,000 rpm for 60 min at 4 C). The pellet was resuspended in RMI, homogenized and Nonidet P-40 (NP-40) was added to 0.05%. The sample was shaken for 15 min on ice and centrifuged in a type 45ti rotor (40,000 rpm for 60 min at 4 C). The pellet was resuspended in RMI and homogenized. The sample was brought to 0.5 M NaCl and 0.6% NP-40. The sample was shaken on ice for 15 min and centrifuged in a type 45ti rotor (40,000 rpm for 60 min at 4 C). The supernatant was used to assay.

General Assay Conditions. Assays were performed at room temperature in a total volume of 200 μL. The assay buffer consisted of 50 mM Hepes, pH 7.5, 140 mM sucrose, 15 mM $MnCl_2$ and 0.5 mg/mL phosphatidylcholine. All measurements were carried out under the same conditions to allow a direct comparison.

Determination of K_m^{app}. The method was adapted from Kellenberger et al [24]. Briefly, [^{3}H]DPPC (50,000 dpm, 50 Ci/mmole) was aliquoted from a chloroform/methanol stock solution into an Eppendorf tube and dried under nitrogen. Reactions were initiated by adding mixtures of increasing amounts of peptidyl substrates, where the final peptide concentrations ranged from 0.5 to 5 K_m^{app}, to a reaction tube containing 10% DMSO, assay buffer and yeast microsomes. Aliquots of the reaction mixture were quenched after 2, 4, 6 and 8

min and the aqueous phase containing the glycopeptide product was quantified [53, 54]. In order to determine the K_m^{app} for each peptide, a Hanes plot was utilized where the [S]/V was plotted as a function of [S], where [S] is the peptide substrate concentration and V is the enzyme velocity. The x-intercept was equivalent to $-K_M^{app}$.

Biotin Assay Conditions. The conditions for the assay were the same as the previous section except that each 40 μL aliquot was quenched into 20 μL stop buffer (750 mM MES, pH 5.01, 1.2% Triton X-100 and 140 mM sucrose). Each aliquot was pipetted onto two, 1 cm^2 SAM^2 biotin capture membranes and washed with the following solutions: four applications of 100 ml 2 M NaCl and 1% Triton X-100 for five min each, one application of 100 ml 2 M NaCl, 1% Triton X-100 and 1% phosphoric acid for five min and one application of 100 ml 95% ethanol for one min. Radioactivity was determined by scintillation counting

HPLC Reconstitution of Oligosaccharyl Transferase. Each protein sample was assayed within two h after purification or stored at -80 C until needed to minimize the loss of enzymatic activity. Both methods resulted in functional enzyme with no significant loss of activity following freeze/thaw cycle of the enzyme. Protein was quantitated using the BCA Assay. The appropriate amount of protein was combined with 15 μl DMSO, 15 μl of 2 mM peptide (Bz-NLT-Me) in DMSO and assay buffer (50 mM Hepes, pH 7.5, 140 mM sucrose, 1.2% Triton X-100, 0.5

mg/ml phosphatidylcholine and 20 mM $MnCl_2$) to a total volume of 350 μl. Following the addition of DMSO, the tube containing dolichyl-PP-dissaccharide (100,000 dpm, 50 Ci/mmole) was vortexed on high for ten s. The assay buffer and protein sample were added and the sample was gently vortexed for ten s. The reaction was initiated with the addition of peptide substrate. The reaction vial was shaken on a rotary shaker at room temperature for 30 min or 24 h. At the appropriate time, the entire reaction was quenched and extracted using a chloroform:methanol (3:2)/20 mM $MgCl_2$ mixture. The aqueous layer was removed and the organic layer was extracted twice with theoretical upper layer with salt (TUP w/ salt, containing 2.75% chloroform, 44% methanol and 1.55 mM $MgCl_2$). The aqueous fraction of the quenched reaction was dried under a steady stream of N_2. This sample was dissolved in 0.75 ml H2O with 0.1 % TFA, filtered and loaded onto an analytical C_{18} HPLC column. This gradient for this method is as follows: Buffer A was H_2O with 0.1% TFA and buffer B was acetonitrile with 0.1% TFA. From t=0-10 min 15% buffer B, t=10-35 min 15-30% buffer B, 35-40 min 30-70% buffer B, 40-50 min 70% buffer B, 50-55 min 70-15% buffer B, and 55-65 min 15% buffer B. In this assay, the first 30 min were collected at one min intervals and counted by scintillation counting. The radioactive product should elute at t=25 min.

Liposome. Lipids were obtained from Avanti in chloroform and aliquoted to obtain phosphatidylcholine: 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]

in a 1.19:0.13 molar ratio. Phospholipid concentrations were obtained from the manufacturer. The sample was concentrated by rotary evaporation and lyophilized. The phospholipids were resuspended in RM (50 mM Hepes, pH 7.5, 140 mM sucrose and 2.5 mM $MgCl_2$) to a final concentration of ~10 mg/ml. The sample was sonicated (15 s on, 30 s off, for 30 min) and incubated on ice for 2 h followed by centrifugation (38,000 rpm in 70 ti rotor for 25 min at 4 C). The samples were stored at 4 C until used.

Electron Microscopy. Grids were prepared by EM facilities (Caltech and MIT) and loaded with 15 μl sample. The sample settled for 1 min and was then washed off with H_2O, One pipette drop of 1% uranyl acetate was added to the grid and incubated for 30 s. The uranyl acetate was washed off with H_2O and dried with filter paper. The sample was examined using an electron microscope.

First Reconstitution Efforts. An aliquot of 100 μg of each protein to be assayed was thawed on ice. A 6:1 phospholipid to protein ratio (w/w), was used for all reconstitution experiments. The phospholipid was typically dried with rotary evaporation and lyophilized overnight. The lipid was resuspended in water to 40 mg/ml. At time 0, the phospholipid and protein were combined and incubated for one h. Throughout this experiment, the sample was shaken at 4 C. The amount of n-Dodecyl-β-D-maltoside in this solution was calculated by multiplying the percentage detergent (1.5 w:v) and the total volume of solution, and at the end of

one h (time= 1 h), enough bio-beads were added to absorb 2X as much detergent as there was in the solution (1 g of Bio-Beads binds 0.105 g detergent). At t=4 h, the sample was removed and placed in a second tube with an equivalent amount of bio-beads. The initial bio-beads were washed twice with buffer. At t=7 h, the previous step was repeated and the sample was allowed to incubate overnight on the shaker at 4 C. The next morning, the sample was removed from the bio-beads and the beads were again washed with buffer. The entire sample was centrifuged at 50,000 rpm for 2 h at 4° C in the 70.1 ti rotor. The choice of buffer for washing the beads was determined by the components of the starting sample. The Hepes, pH 7.5 was always at 50 mM and the concentration of $MgCl_2$ was always at 10 mM. The proteoliposomes will only pellet at less than 50 mM sucrose. The concentration of sucrose in the buffer used to wash the bio-beads can be determined so that the final sucrose concentration during centrifugation is no higher than 50 mM (centrifuge tube volume was 10 ml)

Following centrifugation, the supernatant was removed and the pellet was resuspended thoroughly in 60 μL RM. In order to assay the complex for activity, the reaction was initiated by adding 40 μL of the proteoliposomes to an Eppendorf tube containing the sugar substrate (100,000 dpm, 50 Ci/mmole), 10 μL DMSO, 5 μL 5 mM NLT, 130 μL assay buffer. After four h, the entire reaction was quenched and extracted using a chloroform:methanol (3:2)/20 mM $MgCl_2$ mixture. The aqueous layer was removed and the organic layer was extracted

twice with TUP w/ salt. The aqueous fraction of the quenched reaction was evaoporated under a steady stream of N_2. This sample was then dissolved in 0.75 ml H_2O with 0.1 % TFA and run through the HPLC gradient described earlier using a C_{18} analytical column. In this assay, the first 30 min were collected at one min intervals and analyzed for radioactivity by scintillation counting.

A second aliquot (10 μL) was removed from the proteoliposomes, run on a 15% SDS-PAGE and transferred to nitrocellulose for western blot. A western blot verified the presence of the protein in the proteoliposomes.

Sucrose Gradient Purification of Reconstitution Assay. In this experiment, 100 μgs of each protein was obtained and thawed on ice. Treatment with lipids and bio-beads followed the method described above with the following exceptions. A total of eight equivalents of bio-beads were used to remove detergent from solution. In addition, there was only one change of bio-beads. After treatment with bio-beads, a tube for the 70.1 ti rotor was layered with sucrose in 2 ml aliquots from the bottom up containing 45%, 25%, and 10% sucrose. The bio-bead treated sample was layered on top of the proteoliposomes. This sample was centrifuged at 50,000 rpm at 4° C in the 70.1 to rotor for 12 h. Distinct bands were visible at both sucrose interfaces. Western blotting revealed that all proteins were present at both interfaces. Previous literature suggests that proteoliposomes are present at the 10-25% sucrose gradient [41]. The

proteoliposomes were removed with a pipette and sucrose in the buffer was diluted with buffer containing 50 mM Hepes, pH 7.5 and 10 mM $MgCl_2$. This sample was centrifuged at 50,000 rpm for 2 h at 4 C in the 70.1 ti rotor and assayed for activity as described above.

Assay of "Solubilized Wbp1p" and Wbp1p in Proteoliposomes. The protein in liposomes was assayed as described above. For the soluble assay, the same protein fraction was used and a 50 μL aliquot was assayed directly.

Modified Protein Purification. The cells were harvested, resuspended in RMI (~60 mL) and homogenized. The fractions were sonicated (7 s. on, 7 s. off, 7 cycles) and centrifuged (40,000 rpm, 30 min, 4° C). The pellet was resuspended in ~120 ml RMI + 500 mM NaCl and homogenized. The fraction was shaken in the cold room for 30 min and centrifuged (40,000 rpm, 30 min, 4° C). The pellet was resuspended in ~60 ml RMI + 1.5% n-Dodecyl-β-D-maltoside and homogenized. The sample was shaken in the cold room for 30 min and centrifuged (40,000 rpm, 60 min, 4 C). The volume of the supernatant was quantitated and incubated overnight at 4 C with 4 ml Ni-NTA resin and imidazole to 20 mM. The slurry was then loaded into a column and the sample was allowed to pass through the resin. The resin was then washed with 1) 40 ml RMI + 1.0% Triton X-100 + 20 mM imidazole and 2) 10 ml RMI + 1.0% Triton X-100 + 50 mM imidazole. The protein was eluted from the resin with RMI + 1.0% Triton X-100 +

500 mM imidazole and collected in two ml aliquots. SDS-PAGE was run and the protein was quantitated by Bicinchoninic Protein Assay (BCA assay, Pierce Co.). The fractions containing the desired protein were pooled and a portion of this sample was assayed for activity.

Purification of Stt3p-Protein A Conjugate. The clone YG469 was obtained from the Aebi lab and plated onto tryptophan minus plates at 30 C. Colonies were present after three days and were grown in 100 ml tryptophan minus LB media overnight at 30 C at 300 rpm. A 17 ml aliquot of overnight culture was used to inoculate six flasks containing 1 liter YPD media. The cultures were incubated overnight until OD-600 was greater than 1.5. The cells were harvested by centrifugation (5,000 rpm, 20 min, 4 C, 16ti rotor) and stored at –80 C until needed.

The Stt3p-Protein A conjugate was purified according to Spirig et al [49]. Briefly, the cells were thawed overnight on ice at 4 C and resuspended in lysis buffer (50 mM Tris-HCl, pH, 7.4, 0.2 M mannitol, 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$ 0.1 mM AEBSF, 0.5 μg/ml Pepstatin A and 0.5 μg/ml Leupeptin). The cells were lysed using a Bead-beater with six one min cycles with one min rest on ice in between each cycle. The mixture was centrifuged (2,000 rpm, 8 min, 4 C, 16ti rotor) and the pellet was resuspended in lysis buffer and centrifuged a second time under identical conditions. The total supernatant from both centrifugations were spun a second time (2,000 rpm, 8 min, 4 C, 16ti

rotor). The supernatant from this centrifugation was spun to sediment the microsome membranes (22,000 rpm, 30 min, 4 C, 45ti rotor). The microsome pellet was resuspended in resuspension buffer (50 mM Tris-HCl, pH, 7.4, 0.2 M mannitol, 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$ 10% glycerol, 0.1 mM AEBSF, 0.5 μg/ml Pepstatin A and 0.5 μg/ml Leupeptin) and homogenized. Triton X-100 was added to a final concentration of 1.0% and the sample was shaken on ice for 30 min The solubilized microsomes were centrifuged (36,000 rpm, 30 min, 4 C, 45ti rotor). The supernatant was loaded onto an equilibrated IgG Sepharose column (Amersham Pharmacia Biosciences) and the flow through loaded a second time through the column. The column was washed with 50 ml resuspension buffer with 1.0% Triton X-100, 10 ml of wash buffer (5 mM ammonium acetate, pH 5.0, 1% Triton and 10% glycerol) and eluted with elution buffer (0.5 M acetic acid, pH 3.5, 1% Triton X-100 and 10% glycerol). The elution fractions were immediately neutralized with 3.0 M Tris, pH, 7.5. The protein was quantitated using a BCA Assay and verified by Western blot with rabbit anti-goat antibody conjugated to alkaline phosphatase using the NBT/BCIP substrate.

Purification of Co-Expression of Proteins in Insect Cells. A one liter aliquot of Sf9 cells, at a density of 3.0×10^6 cells/ml was infected with a M.O.I. of 5 for the Nlt1p, Ost2p, Swp1p and Wbp1p viruses. The cells were harvested at 72-96 h post-

infection when cell viability was 50-80%. The cells were centrifuged (8,000 rpm, 30 min, 4 C, 16ti rotor) and the cell pellets were frozen at –80 C until required.

The cells were thawed overnight on ice at 4 C and sonicated in RMI (50 mM Hepes, pH, 7.5, 140 mM sucrose, 10 mM $MgCl_2$, 0.1 mM AEBSF, 0.5 μg/ml Pepstatin A and 0.5 μg/ml Leupeptin) for seven cycles of seven s with the same time of rest in between on ice. The cells were shaken on ice for 30 min and centrifuged (25,000 rpm, 30 min, 4 C, 28ti rotor). The cell pellet was subsequently homogenized in RMI plus 0.5 M NaCl and shaken on ice for 30 min. The sample was centrifuged (25,000 rpm, 30 min, 4 C, 28ti rotor) and the pellet was resuspended in RMI and homogenized. n-Dodecyl-β-maltoside was added to a final concentration of 1.5% and the sample was shaken on ice for 30 min. The sample was centrifuged (25,000 rpm, 30 min, 4 C, 28ti rotor) and imidazole was added to the supernatant to a final concentration of 15 mM. The protein was loaded onto an equilibrated Ni-NTA (Qiagen, Inc.) column and subsequently washed with 40 ml of RMI with 1% Triton X-100 and 20 mM imidazole, then eluted with RMI with 1% Triton and 500 mM imidazole. The protein was quantitated using a BCA assay and the protein was verified by western blot using a FLAG antibody.

Pulldown experiment with Stt3p-Protein A conjugate and Nlt1p/Ost2p/Swp1p/ Wbp1p co-expression. A 100 μg sample of Stt3p-Protein A, Nlt1p/Ost2p, Swp1p and Wbp1p with BSA was loaded onto a IgG-Sepharose column. The flow

through sample was collected and the column was washed with 10 ml resuspension buffer, 10 ml wash buffer and eluted with elution buffer. The samples were loaded onto 15% SDS-PAGE and probed with either a goat α-rabbit secondary antibody for Stt3p-Protein A or a FLAG monoclonal antibody with an alkaline-phosphatase secondary antibody. All proteins were detected with NBT/BCIP developer (Pierce Co.).

III-16 References

1. Peluso, S., Ufret, M., O'Reilly, M. & Imperiali, B. (2002) Neoglycopeptides as inhibitors of oligosaccharyl transferase: Insight into negotiating product inhibition, *Chem. Biol. 9*, 1323-1328.
2. Stagljar, I., Korostensky, C., Johnsson, N. & te Hessen, S. (1998) A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo, *Proc. Natl. Acad. Sci. U.S.A. 95*, 5187-5192.
3. Yan, Q. & Lennarz, W. (2002) Studies on the function of oligosaccharyl transferase subunits: A glycosylatable photoprobe binds to the luminal domain of Ost1p, *Proc. Natl. Acad. Sci. U.S.A. 99*, 15994-15999.
4. Pathak, R., Parker, C. & Imperiali, B. (1995) The essential yeast NLT1 gene encodes the 64 kDa glycoprotein subunit of the oligosaccharyl transferase, *FEBS Lett. 362*, 229-234.

5. Knauer, R. & Lehle, L. (1999) The oligosaccharyltransferase complex from *Saccharomyces cerevisiae*: Isolation of the Ost6p gene, its synthetic interaction with Ost3p, and analysis of the native complex, *J. Biol. Chem. 274*, 17249-17256.

6. Pathak, R. & Imperiali, B. (1997) A dual affinity tag on the 64-kDa Nlt1p subunit allows the rapid characterization of mutant yeast oligosaccharyl transferase complexes, *Arch. Biochem. Biophys. 338*, 1-6.

7. Kelleher, D. & Gilmore, R. (1994) The *Saccharomyces cerevisiae* oligosaccharyltransferase is a protein complex composed of Wbp1p, Swp1p, and 4 additional polypeptides, *J. Biol. Chem. 269*, 12908-12917.

8. Knauer, R. & Lehle, L. (1999) The oligosaccharyltransferase complex from yeast, *Biochim. Biophys. Acta. 1426*, 259-276.

9. te Hessen, S., Rauhut, R., Aebersold, R., Abelson, J., Aebi, M. & Clark, M. (1991) An essential 45 kDa yeast transmembrane protein reacts with anti-nuclear pore antibodies: purification of the protein, immunolocalization and cloning of the gene, *Eur. J. Cell Biol. 56*, 8-18.

10. Dempski, R. & Imperiali, B. (2002) Oligosaccharyl transferase: gatekeeper to the secretory pathway, *Curr. Opin. Chem. Biol. 6*, 844-850.

11. Karaoglu, D., Kelleher, D. & Gilmore, R. (1995) Functional characterization of Ost3p. Loss of the 34-kD subunit of the *Saccharomyces cerevisiae* oligosaccharyltransferase results in biased underglycosylation of acceptor substrates, *J. Cell Biol. 130*, 567-577.

12. te Hessen, S., Knauer, R., Lehle, L. & Aebi, M. (1993) Yeast Wbp1p and Swp1p form a protein complex essential for oligosaccharyl transferase activity, *EMBO J. 12*, 279-284.

13. Silberstein, S., Collins, P., Kelleher, D. & Gilmore, R. (1995) The essential Ost2 gene encodes the 16-KD subunit of the yeast oligosaccharyltransferase, a highly conserved protein expressed in diverse eukaryotic organisms, *J. Cell Biol. 131*, 371-383.

14. McIntire, W., Myung, C., MacCleery, G., Wang, Q. & Garrison, J. (2002) Reconstitution of G protein-coupled receptors with recombinant G protein alpha and beta gamma subunits, *Methods Enzymol. 343*, 372-393.

15. Mikuni, O., Trager, J., Ackerly, H., Weinrich, S., Asai, A., Yamashita, Y., Mizukami, T. & Anazawa, H. (2002) Reconstitution of telomerase activity utilizing human catalytic subunit expressed in insect cells, *Biochem. Biophys. Res. Commun. 298*, 144-150.

16. Bozon, W., Couture, L., Pajot-Augy, E., Richard, F., Remy, J. & Salesse, R. (2002) Rescue of intracellularly trapped lutropin receptor exodomain by endodomain and reconstitution of a functional membrane receptor: Interaction between exo- and endodomains, *Protein Expres. Purif. 25*, 114-123.

17. Nemeth, J., Hochensang, G., Marnett, L. & Caprioli, R. (2001) Characterization of the glycosylation sites in cyclooxygenase-2 using mass spectrommetry, *Biochemistry. 40*, 3109-3116.

18. O'Connor, S. (2001) *Conformational effects of asparagine-linked glycosylation*, Massachusetts Institute of Technology, Cambridge.

19. Wyss, D. & Wagner, G. (1996) The structural role of sugars in glycoproteins, *Prot. Eng. 7*, 409-416.

20. Rudd, P., Joao, H., Coghill, E., Fiten, P., Saunders, M., Opdenakker, G. & Dwek, R. (1994) Glycoforms modify the dynamic stability and functional activity of an enzyme, *Biochemistry. 33*, 17-22.

21. Tierney, M. & Unwin, N. (2000) Electron microscopic evidence for the assembly of soluble pentameric extracellular domains of the nicotinic acetylcholine receptor, *J. Mol. Biol. 303*, 185-196.

22. Heifetz, A., Keenan, R. & Elbein, A. (1979) Mechanism of action of tunicamycin on the UDP-GlcNAc-dolichyl-phosphate GlcNAc-1-phosphate transferase, *Biochemistry. 18*, 2186-2192.

23. Knauer, R. & Lehle, L. (1999) The oligosaccharyltransferase complex from yeast, *Biochim. Biophys. Acta. 1426*, 259-273.

24. Kellenberger, C., Hendrickson, T. & Imperiali, B. (1997) Structural and functional analysis of peptidyl oligosaccharyl transferase inhibitors, *Biochemistry. 36*, 12554-12559.

25. Eason, P. & Imperiali, B. (1999) A potent oligosaccharyl transferase inhibitor that crosses the intracellular endoplasmic reticulum, *Biochemistry. 38*, 5430-5437.

26. Selo, I., Negroni, L., Creminon, C., Grassi, J. & Wal, J. (1996) Preferential labeling of alpha-amino N-terminal groups in peptides by biotin, application to the detection of specific anti-peptide antibodies by enzyme immunoassays, *J. of Immun. Meth. 199*, 127-138.

27. Imperiali, B., Shannon, K. & Rickert, K. (1992) Role of peptide conformation in asparagine-linked glycosylation, *J. Am. Chem. Soc. 114*, 7942-7944.

28. Imperiali, B., Shannon, K., Unno, M. & Rickert, K. (1992) A mechanistic proposal for asparagine-linked glycosylation, *J. Am. Chem. Soc. 114*, 7944-7945.

29. Srinivasan, A. & Coward, J. (2002) A biotin capture assay for oligosaccharyltransferase, *Ann. Biochem. 306*, 328-335.

30. Czerski, L. & Sanders, C. (2000) Functionality of a membrane protein in bicelles, *Ann. Biochem. 284*, 327-333.

31. Nagel, G., Ollig, D., Fuhrmann, M., Kateriya, S., Mustl, A., Bamberg, E. & Hegemann, P. (2002) Channelrhodopsin-1: A light-gated proton channel in green algae, *Science. 296*, 2395-2398.

32. Turina, P., Samoray, D. & Graber, P. (2003) H+/ATP ratio of proton transport-coupled ATP synthesis and hydrolysis catalysed by CF_0F_1-liposomes, *EMBO J. 22*, 418-426.

33. Putman, M., van Veen, H. & Konings, W. (2000) Molecular properties of bacterial multidrug transporters, *Microbiology and Molecular Biology Review. 64*, 672.

34. Walter, A., Kuehl, G., Barnes, K. & VanderWaerdt, G. (2000) The vesicle-to-micelle transition of phosphatidylcholine vesicles induced by nonionic detergents: effects of sodium chloride, sucrose and urea, *Biochim. Biophys. Acta. 1508*, 20-33.

35. Rigaud, J., Levy, D., Mosser, G. & Lambert, O. (1998) Detergent removal by non-polar polystyrene beads-Applications to membrane protein reconstitution and two-dimensional crystallization, *Eur. Biophys. J. 27*, 305-319.

36. Allen, T., Romans, A., Kercet, H. & Segrest, J. (1980) Detergent removal during membrane reconstitution, *Biochim. Biophys. Acta. 601*, 328-342.

37. Knol, J., Veenhoff, L., Liang, W., Henderson, P., Leblanc, G. & Poolman, B. (1996) Unidirectional reconsitution into detergent-solubilized liposomes of the purified lactose transport system of *Streptococcus thermophilus*, *J. Biol. Chem. 271*, 15358-15366.

38. Knol, J., Sjollema, K. & Poolman, B. (1998) Detergent-mediated reconstitution of membrane proteins, *Biochemistry. 37*, 16410-16415.

39. Fang, G., Friesen, R., Lanfermeijer, F., Hagting, A., Poolman, B. & Konings, W. (1999) Manipulation of activity and orientation of membrane-reconstituted di-tripeptide transport protein DtpT of *Lactococcus lactis*, *Mol. Memb. Biol. 16*, 297-304.

40. Rigaud, J., Pitard, B. & Levy, D. (1995) Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins, *Biochim. Biophys. Acta. 1231*, 223-246.

41. DeGrip, W., VanOostrum, J. & Bovee-Geurts, P. (1998) Selective detergent-extraction from mixed detergent/lipid/protein micelles, using cyclodextrin inclusion compounds: a novel generic approach for the preparation of proteoliposomes, *Biochemical J. 330*, 667-674.

42. Scheiner-Bobis, G. (2002) The sodium pump-Its molecular properties and mechanics of ion transport, *Eur. J. Biochem. 269*, 2424-2433.

43. Geibel, S., Kaplan, J., Bamberg, E. & Friedrich, T. (2003) Conformational dynamics of the Na^+/K^+-ATPase probed by voltage clamp fluorometry, *Proc. Natl. Acad. Sci. U.S.A. 100*, 964-969.

44. Pathak, R., Hendrickson, T. & Imperiali, B. (1995) Sulfhydryl modification of the yeast Wbp1p inhibits oligosaccharyl transferase activity, *Biochemistry. 34*, 4179-4185.

45. Yoshida, S., Ohya, Y., Nakano, A. & Anraku, Y. (1995) Stt3, a novel essential gene-related to the PKC1/STT1 protein-kinase pathway, is involved in protein glycosylation in yeast, *Gene. 164*, 167-172.

46. Zufferey, R., Knauer, R., Burda, P., Stagljar, I., te Hessen, S., Lehle, L. & Aebi, M. (1995) Stt3, a highly conserved protein required for yeast oligosaccharyl transferase activity in-vivo, *EMBO J. 14*, 4949-4960.

47. Wackler, M., Linton, D., Hitchen, P., Nita-Lazar, M., Haslam, S., North, S., Panico, M., Morris, H., Dell, A., Wren, B. & Aebi, M. (2002) N-linked protein glycosylation in Campylobacter jejuni and its functional transfer into E. coli, *Science. 298*, 1790-1793.

48. Yan, Q. & Lennarz, W. (2002) Studies on the function of oligosaccharyl transferase subunits: Stt3p is directly involved in the glycosylation process, *J. Biol. Chem. 277*, 47692-47700.

49. Spirig, U., Glaras, M., Bodmer, D., Reiss, G., Burda, P., Lippuner, V., te Hessen, S. & Aebi, M. (1997) The Stt3 protein is a component of the yeast oligosaccharyltransferase complex, *Mol. Gen. Genet. 256*, 628-637.

50. Luckow, V. & Summers, M. (1988) Trends in the development of baculovirus expression vectors, *Bio/Technology. 6*, 47-55.

51. Tessier, D., Thomas, D., Khouri, H., Laliberte, F. & Vernet, T. (1991) Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide, *Gene. 98*, 177-183.

52. O'Reilly, D., Miller, L. & Luckow, V. (1994) *Baculovirus expression vectors: A laboratory manuel*, Oxford University Press, New York.

53. Imperiali, B. & Shannon, K. (1991) Differences between Asn-Xaa-Thr-containing peptides-A comparison of solution conformation and substrate behavior with oligosaccharyltransferase, *Biochemistry. 30*, 4374-4380.

54. Sharma, C., Lehle, L. & Tanner, W. (1981) N-glycosylation of yeast proteins-Characterization of the solubilized oligosaccharyl transferase, *Eur. J. Biochem. 116*, 101-108.

55. Atherton, E. & Sheppard, R. C. (1989) *Solid phase peptide synthesis*, Oxford University Press, New York.

56. Virgilio, A. & Ellman, J. (1994) Simultaneous solid-phase synthesis of beta-turn mimetics incorporating side-chain functionality, *J. Am. Chem. Soc. 116*, 11580-11581.

57. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. & Gray, T. (1996) How to measure and predict the molar absorption coefficient of a protein, *Protein Sci. 4*, 2411-2423.

Chapter III: Characterization of the Soluble Domains of Three Essential Proteins from Oligosaccharyl Transferase

[Part of this chapter has been submitted as: Dempski RE, Imperiali B. The effect of glycosylation on protein stability of oligosaccharyl transferase. Arch. Biochem. Biophys.]

III-1 Introduction

The elusiveness of oligosaccharyl transferase is partially mediated by the fact that all of the subunits in this enzyme are membrane proteins. This results in proteins which are difficult to isolate and are present in low concentration. Due to these issues, we were interested in expressing the soluble domains of three of the essential proteins (Nlt1p, Swp1p and Wbp1p). Expression of the soluble domains of these proteins in a heterologous system would enable purification of high concentrations of tractable proteins which could potentially be studied using a variety of biophysical techniques. In order for these studies to be successful, the proteins must be well-behaved and post-translationally processed in a manner similar to that of the native subunits in *S. cerevisiae*. Finally, these studies provide the framework for reconstitution of the soluble domains of a multimeric membrane bound enzyme complex to form a catalytically competent macromolecular structure, an endeavor which has not yet been accomplished for any analogous enzyme.

The lytic baculovirus/insect cell expression system has proved advantagous in a variety of protein systems since it is capable of expressing high

concentrations of properly folded proteins that pass through the secretory pathway. The four soluble domains of the nicotinic acetylcholine receptor have been expressed in insect cells and have been determined to be properly processed, glycosylated and to form native contacts [1]. In addition, this protein expression system has been used to reconstitute a variety of enzymes such as G-protein coupled receptors, telomerases, the membrane-bound lutropin receptor and cyclooxygenase-2 [2-4] [5].

In this chapter, three constructs, which include the luminal domains of three of the essential proteins of oligosaccharyl transferase have been expressed and purified in the lytic baculovirus/insect cell expression system. These proteins are well behaved in solution, structured and are heterogeneously glycosylated. In both glycosylated proteins, the oligosaccharide moieties appear to have a significant effect on the global structure of the proteins as observed by circular dichroism. In addition, two of the proteins appear to be stabilized when co-expressed and this stabilization is abolished when the oligosaccharides are removed from the protein.

III-2 Expression and Purification of Constructs

Due to the complexity of the system and the difficulty of obtaining large amounts of catalytically-competent enzyme, it has been challenging to investigate the protein-protein associations of the enzyme. To this end, mutants

were constructed to investigate the soluble domains of three essential subunits: Nlt1p, Swp1p and Wbp1p. The three recombinant proteins were originally expressed in the methylotropic yeast, *P. pastoris*, but the protein was secreted in low levels and was mostly present as insoluble protein aggregates (data not shown).

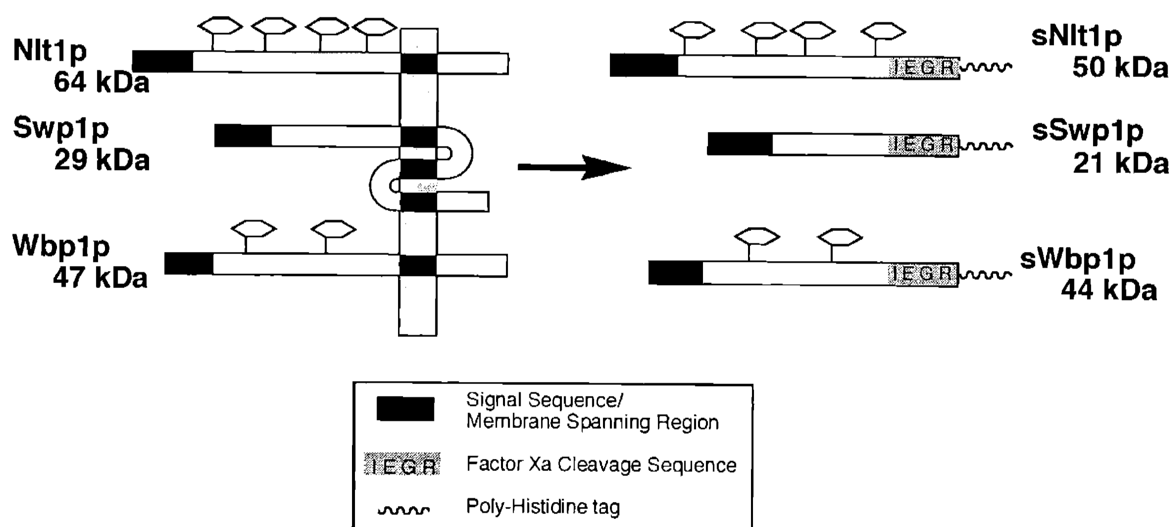


Figure III-1. Schematic of the three oligosaccharyl transferase constructs including N-linked glycosylation sites and introduction of Factor Xa cleavage sequence and poly-histidine tag.

The proteins were subsequently expressed in baculovirus-infected insect cells. The three constructs included a Factor Xa cleavage site and a poly-histidine tag on the C-terminus (Figure III-1). A summary of the recombinant constructs, named sNlt1p, sSwp1p and sWbp1p, is included in Table III--1. The constructs are numbered according to the initial cloning of these proteins [6-8]. The constructs encoding sNlt1p and sSwp1p used the pVL1393 vector since these proteins retained their native signal sequence, while the construct encoding sWbp1p used the pVT-Bac vector and utilized a non-native signal

sequence. N-terminal sequencing verified proper processing of these proteins. Table III-1 includes the results of N-terminal sequencing and the number of glycosylation sites predicted for each protein from the primary sequence.

Table III-1. A summary of the constructs of oligosaccharyl transferase in this study.

	Vector	Residues	Predicted Molecular Weight (kD)	Predicted N-terminal Sequence	N-terminal Amino Acid Sequencing of Protein	No. of Potential N-linked Glycosylation Sites
sNlt1p	pVL1393	1-452	50.0	AQYEP...	AQYEPPAT...	Up to 4
sSwp1p	pVL1393	1-195	20.8	YVAQD...	YVAQDVHV...	0
sWbp1p	pVT-Bac	21-389	44.4	DPSPG...	DPSPGERT...	Up to 2

Following virus infection, optimal expression with minimal protein degradation was observed when the cells were harvested with a viability of 60-80%, 72-96 hours post-infection using a multiplicity of infection (M.O.I.) of 5. Recombinant protein was observed to be both secreted into the media and to be present in the cells upon harvest. The proteins could be isolated and purified to homogeneity using a one-step Ni-NTA purification procedure (Figure III-2). Protein yields were greater than 3.0 mg/L culture for each protein

The glycosylation states of both sNlt1p and sWbp1p were examined through the use of tunicamycin. Tunicamycin inhibits the transfer of GlcNAc-P from UDP-GlcNAc to dolichol phosphate, which is the first step in the synthesis of the dolichol pyrophosphate oligosaccharide donor [9]. Insect cells were infected with the appropriate virus with tunicamycin at a final concentration of 1.0 μg/ml.

Protein was isolated 72 hours post-infection and run on 8% SDS-PAGE to resolve the glycosylation state of the proteins. This method has previously been used to determine the glycosylation state of soluble domains of the nicotinic acetylcholine receptor in insect cells [1]. Both sNlt1p and sWbp1p were observed to be glycosylated (Figure III-3). sNlt1p lacks distinct bands, while sWbp1p has two distinct bands. For both proteins, addition of tunicamycin collapsed the bands into one discrete band as seen by western blot analysis.

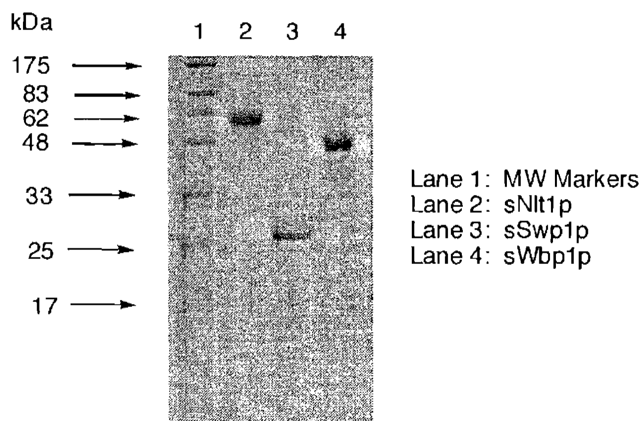


Figure III-2. Coomassie Blue stain of a 15% SDS-PAGE of the three recombinant proteins expressed and purified from the lytic baculovirus/insect cell expression system.

III-3 Determination of the aggregation state of the proteins in solution

Previous research has suggested numerous protein/protein interactions within the oligosaccharyl transferase enzyme complex by immunoprecipitation as well as biochemical and genetic studies [7, 10-13]. Since the proteins were being examined without their transmembrane domains, it was unknown if these

proteins could maintain a monomeric state in solution. Following protein purification, gel filtration chromatography was used to determine the oligomeric state of the proteins (Figure III-4). The protein sNlt1p was observed to have a molecular weight of 59.4 kD, a mass that is consistent with a monomeric glycosylated protein (Table III-2). The molecular weight of sWbp1p was determined to be 47.7 kDa and is consistent with the mass of a monomeric glycoprotein.

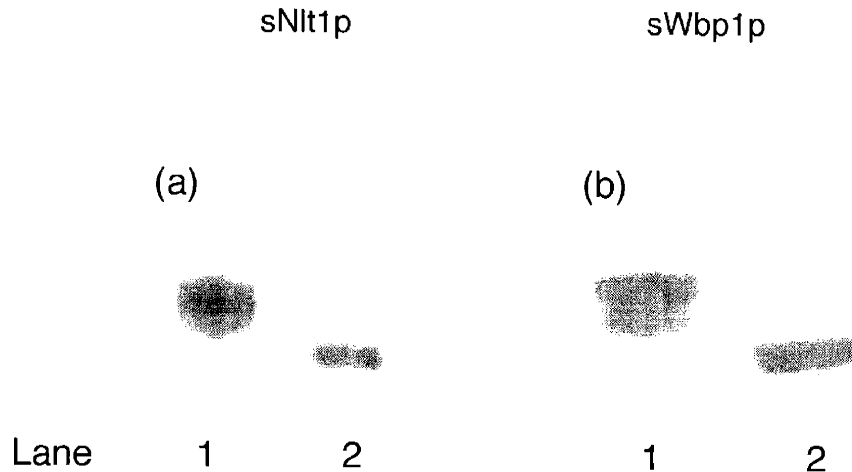


Figure III-3. Western blot demonstrating the glycosylation state of recombinant (a) sNlt1p and (b) sWbp1p proteins expressed and purified in Sf9 cells. Lane 1 is protein expression under normal conditions. Lane 2 is protein expression in the presence of 1.0 μg/ml tunicamycin.

The protein sSwp1p has proven more difficult to analyze. The mass of the protein was determined to be 36.3 kDa by gel filtration chromatography. Mass spectral analysis of the protein suggested a molecular weight of 20,868 Da (data not shown), which corresponds to the protein plus formic acid, which was in the sample preparation. In order to further probe the state of the protein, analytical

ultracentrifugation was employed. Samples were prepared under denaturing and native conditions. A summary of the data is shown in Table III-3. The data was fit to a single ideal species using the program WinNonlin V1.08 to obtain sigma and the sigma values for the 95% confidence interval [14]. The density of the buffers and the protein molecular weight was determined using the program Sednterp [15, 16]. The protein was a single species under both native and denaturing conditions.

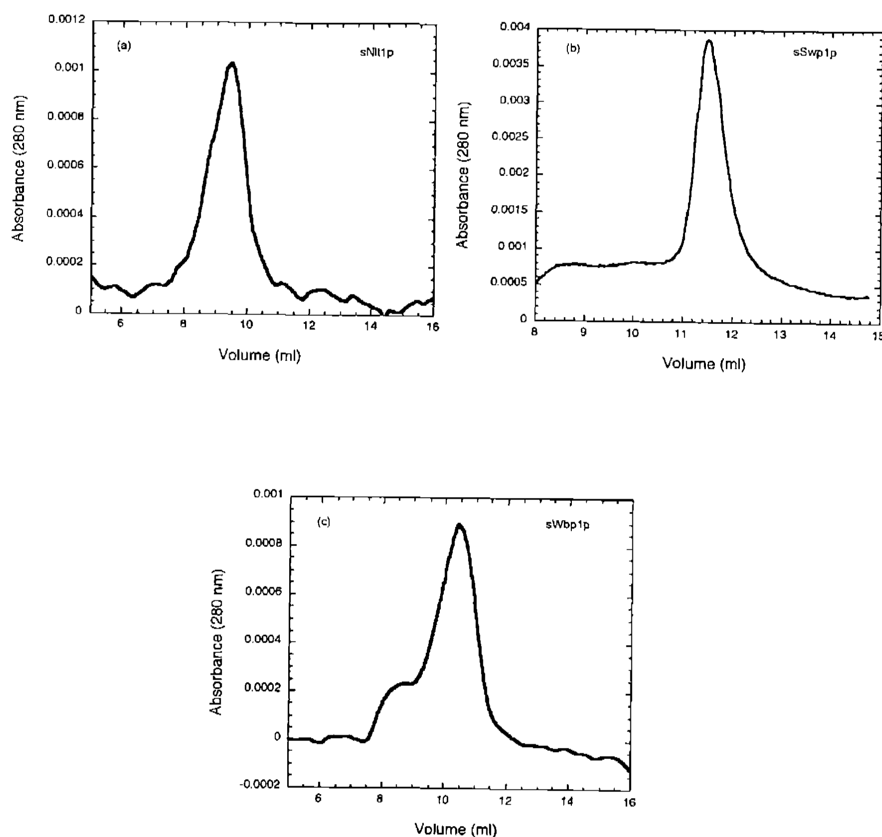


Figure III-4. A summary of the gel filtration traces of (a) sNlt1p, (b) sSwp1p and (c) sWbp1p. The data were obtained on an Amersham-Pharmacia Biotech FPLC monitoring protein elution at 280 nm at room termperature. All experiments were in the presence of 50 mM Tris, pH 7.5 and 2.5 mM $MgCl_2$.

Under native conditions, the molecular weight of the protein was determined to be 26.2 kDa, while under denaturing conditions the mass of the protein was calculated to be 23.9 kDa, close to the monomeric weight of the protein. The larger than expected mass could be due to nonideal behavior [17].

Table III-2. A summary of the elution profiles from the gel filtration chromatography of the recombinant proteins as shown in Figure III-2. A standard curve was generated using molecular weight standards from Amersham-Pharmacia Biotech and plotted as K_{av} vs. Molecular Weight.

	Predicted Protein Molecular Weight (kDa)	No. of Glycosylation Sites from Primary Sequence	Elution Volume (min)	Calculated Molecular Weight (kDa)
sNlt1p	50.0	up to 4	9.46	59.4
sSwp1p	20.8	0	11.51	36.3
sWbp1p	44.4	up to 2	10.50	47.7

III-4 Structural analysis of the three soluble domains

No structural data has been presented to date for the proteins comprising the oligosaccharyl transferase. Circular dichroism spectroscopy was used to obtain insight into the secondary structure content of these proteins in the presence and absence of divalent metal ions (Mg^{2+}). Similar biophysical studies were carried out on the unglycosylated proteins (Figure III-5). There was no significant secondary structure change in the presence or absence of magnesium for sNlt1p and sSwp1p. This suggests that although the enzyme requires a divalent metal for activity, the structure of neither protein is affected by divalent metal ions [18]. In contrast, the protein sWbp1p precipitated out of solution in the

absence of metal ions, suggesting that this protein might play an important role in metal binding for this enzyme.

Table III-3. A summary of the molecular weight of sSwp1p as obtained by analytical ultracentrifugation.

	Sigma (95% Confidence Interval)	Molecular Weight (Da) (95% Confidence Interval)
11.31 μM sSwp1p 6M Guanidine HCl 50 mM sodium phosphate, pH, 7.5	1.90 (1.66-2.13)	26,248 (22,940-29,466)
11.31 μM sSwp1p 50 mM sodium phosphate, pH 7.5 150 mM sodium chloride 1 mM magnesium chloride	2.74 (2.11-3.43)	23,872 (18,354-29,885)

In order to further examine the role of divalent metals with this protein, inductively coupled plasma-emission spectrometry (ICP-ES) was employed to determine the stoichiometry of metal ions associated with this protein [19, 20]. In comparison to sNlt1p (0.03 parts Mg^{2+} per part protein), Mg^{2+} was found to associate with sWbp1p at 0.63 parts Mg^{2+} per part protein. Neither Nlt1p nor Wbp1p appeared to bind Mn^{2+}, as determined by ICP-MS. This supports prior studies that proposed that the protein Wbp1p is important in catalysis. Experiments have also shown that when the enzyme complex is incubated with the labeling reagent S-[(N-biotinoylamino)ethyl] methanethiolsulfonate (BMTS) the enzyme is inactivated and the protein Wbp1p is labeled. This inactivation is

reversible and can be prevented with the pre-incubation of the substrate (dolichylpyrophosphoryl)-N,N'-diacetylchitobiose (Dol-PPGlcNAc$_2$) [21].

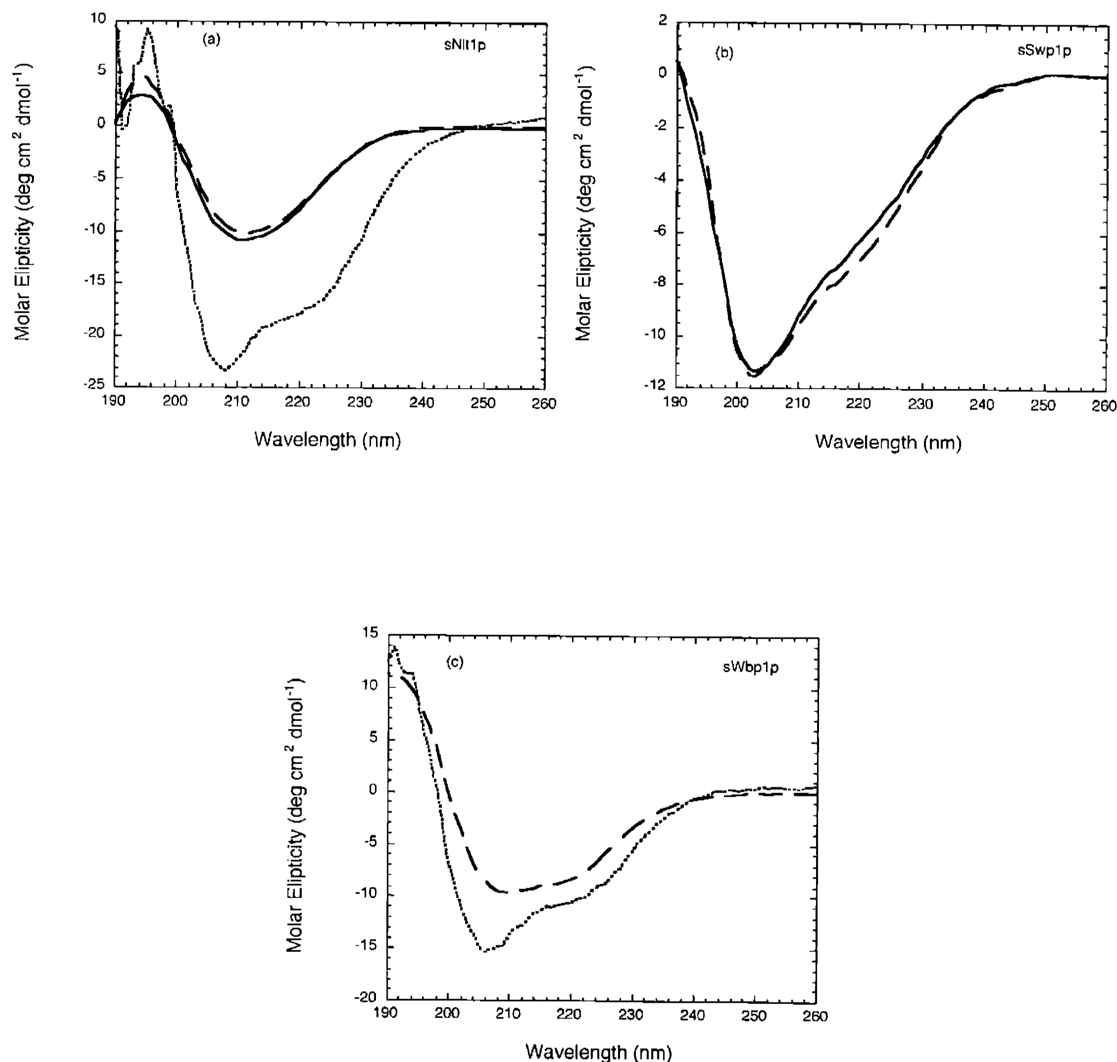


Figure III-5. Circular Dichroism Analysis of (a) sNlt1p, (b) sSwp1p and (c) sWbp1p at 4 C in 50 mM sodium phosphate, pH 7.5. The (–) represents protein with no magnesium chloride. The (— —) line represents protein with 5 mM magnesium chloride and the (··········) line displays protein expressed in the presence of tunicamycin with 50 mM sodium phosphate, pH 7.5 and 5 mM magnesium chloride.

Both sNlt1p and sWbp1p demonstrated significant global structural changes when the proteins were expressed in the presence of tunicamycin. In order to obtain a more quantitative analysis of the secondary structure of these proteins, the program k2D was employed, which uses a Kohonen neural network with a 2-dimensional output layer to determine the secondary structure of proteins from circular dichroism data [22, 23]. A summary of the data generated from this algorithm is presented in Table III-4.

Table III-4. A summary of the secondary structure elements of sNlt1p, sSwp1p and sWbp1p using the neural network program k2D.

Protein		α Helix (%)	β Sheet (%)	Random Coil (%)
sNlt1p	w/o $MgCl_2$	24	40	36
	w/ $MgCl_2$	24	40	37
	w/ Tunicamycin	56	9	35
sSwp1p	w/o $MgCl_2$	10	35	56
	w/ $MgCl_2$	13	32	56
	w/ Tunicamycin	N/A	N/A	N/A
sWbp1p	w/o $MgCl_2$	N/A	N/A	N/A
	w/ $MgCl_2$	24	39	36
	w/ Tunicamycin	28	18	54

For the protein sNlt1p, there appears to be a significant amount of both α-helix and β-sheet character, at 24% and 40% respectively. The loss of glycosylation of this protein dramatically increases the percentage of α-helix (24% to 56%) and decreases the percentage of β-sheet (40% to 9%). These data suggest that glycosylation is important to the global fold of this protein. In order to probe whether glycosylation is important in the stability of sNlt1p, the

unfolding of the proteins was monitored at 222 nm as a function of temperature (Figure III-6). Both glycosylated and unglycosylated sNlt1p appear to have a melting temperature of approximately 60 C, suggesting that although the secondary structure is affected by N-linked glycosylation, the overall stability of sNlt1p is unaffected by the loss of oligosaccharides.

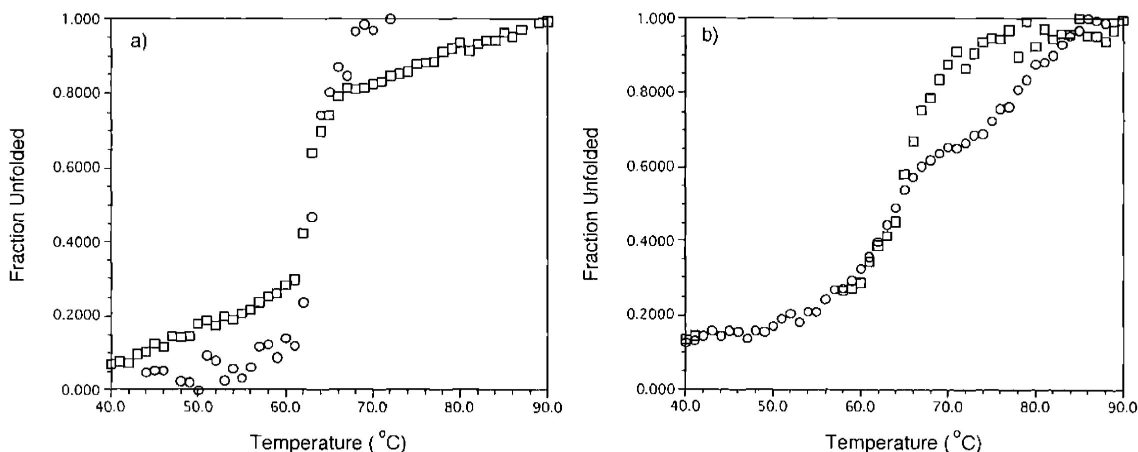


Figure III-6. A Comparison of the thermal stability of sNlt1p (a) and sWbp1p (b) as measured at 222 nm using an Aviv Circular Dichroism Spectrometer Model 202. The (oooo) represents protein expressed under native conditions, while the (□□□□) represents protein expressed in the presence of 1.0 μg/ml tunicamycin.

The protein sSwp1p appears to have more β-sheet character than α-helix character with a significant amount of random coil in the structure, with 32%, 13% and 56%, respectively of each element. No significant change in secondary structure is observed in the presence or absence of magnesium chloride, suggesting that the metal ion plays little or no role in the structure of this protein.

The protein sWbp1p demonstrates significant α-helix (24%) and β-sheet (38%) character. As discussed, it is important to note that the protein is not soluble in buffer deficient in divalent metal ions. In addition, the secondary structure of the protein is altered when the protein is expressed in the presence of tunicamycin. This is demonstrated by a change in β-sheet character (39% to 18%) and an increase in random coil from 36% to 54%. Similar to sNlt1p, the thermal stability of sWbp1p is not affected by glycosylation (Figure III-6b). The melting temperature for both subunits is approximately 63 C. For both sNlt1p and sWbp1p, glycosylation appears to affect the secondary structure of the protein, but not the overall stability of the constructs.

III-5 A study of protein/protein interactions between the soluble domains

A variety of studies have focused on examining interactions between proteins in the oligosaccharyl transferase complex. From these biochemical experiments, three subcomplexes have been identified, Nlt1p/Ost5p, Swp1p/Wbp1p/Ost2p and Stt3p/Ost4p/Ost3p [7, 10-13, 24]. In addition, protein-protein interactions have been observed between proteins in different subcomplexes. For example a split-ubiquitin experiment revealed an interaction between Nlt1p and Wbp1p [25]. In addition, OST48, a mammalian homolog to Wbp1p, was seen to interact with ribophorin I and II, the mammalian homologs of Nlt1p and Swp1p, respectively [26]. These data suggest that all of the proteins

from this complex are within close proximity and subunits from different subcomplexes interact with each other.

In order to examine whether the three soluble proteins interact with each other, the proteins were co-expressed in sets of two, co-purified and analyzed by circular dichroism thermal denaturation (Figure III-7). This technique has been used previously to examine the interaction of smooth-muscle calponin-caltropin [27]. In Figure III-7a, the T_m of the co-expression of sSwp1p and sWbp1p was determined to be 58 C, which is approximately halfway between the transition temperature for each individual protein, 54 C for sSwp1p and 65 C for sWbp1p. In Figure III-7b, the thermal transition temperature of sNlt1p was 63 C and the melting temperature of sWbp1p was determined to be 65 C. The melting temperature of these two proteins when co-expressed was 64 C, suggesting no thermal stabilization when these two proteins are associated.

The impetus for this set of experiments was the observation that sNlt1p and sSwp1p could not be expressed separately and combined without protein precipitation despite the use of multiple pH and salt conditions. This suggested that the proteins were interacting with each other. In contrast, the proteins remained stable when co-expressed and purified. In order to further probe this interaction, sNlt1p and sSwp1p were expressed in a 1:1 stoichiometry as observed by SDS-PAGE and were examined using thermal denaturation (Figure III-7c). The transition temperature of sNlt1p resulted in a T_m of 63 C and the T_m of sSwp1p was determined to be 54 C. The transition temperatures of these

proteins was observed to shift to a higher transition temperature (73 C) when the two proteins were-co-expressed that sNltp1p and sSwp1p are stabilized during co-expression.

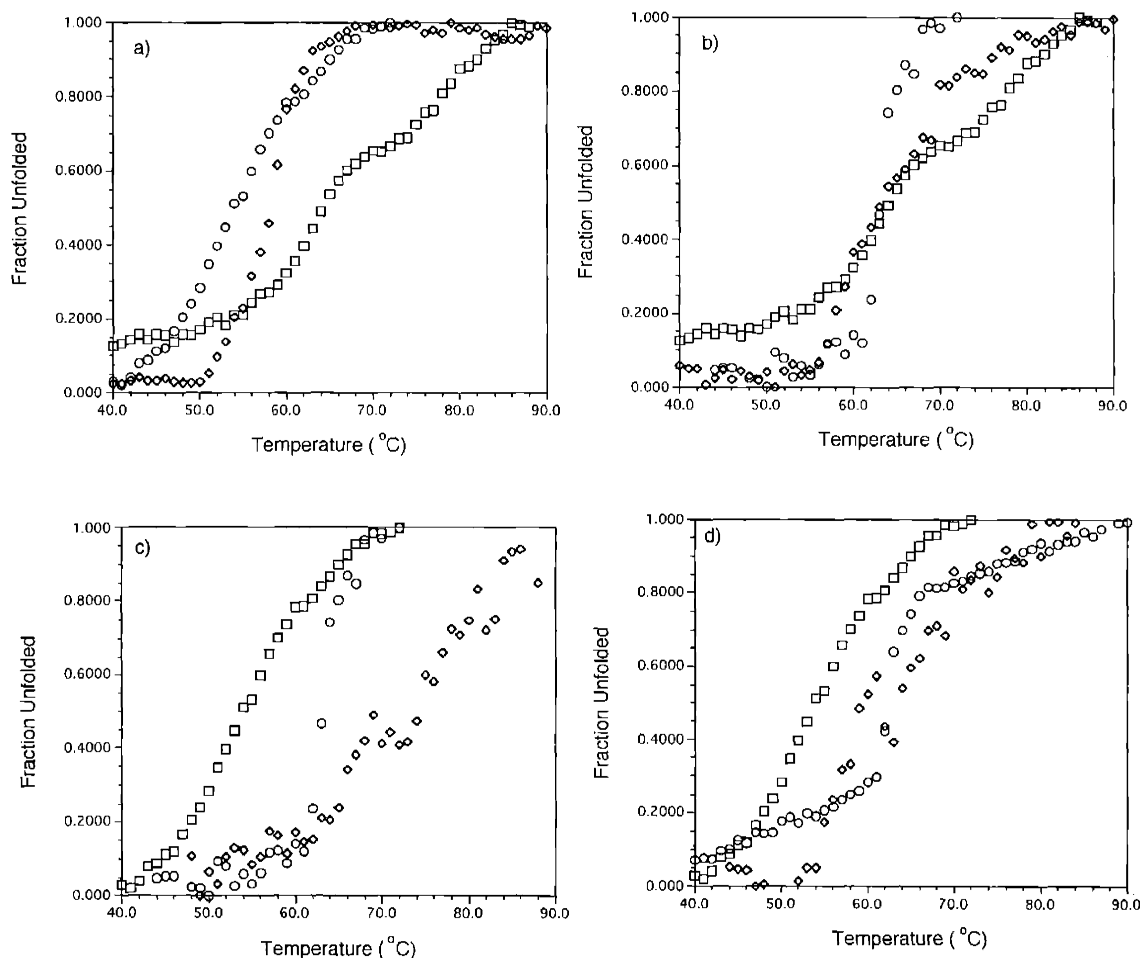


Figure III-7. Thermal denaturation of recombinant oligosaccharyl transferase subunits using circular dichroism, measured at 222 nm. In (a), the (○○○○) is sSwp1p, the (□□□□) sWbp1p and the co-expression of sSwp1p and sWbp1p is (◊◊◊◊). In (b), (○○○○) are sNlt1p, the (□□□□) are sWbp1p and the (◊◊◊◊) are the co-expression of sNlt1p and sWbp1p. In (c), (○○○○) are sNlt1p, the (□□□□) are sSwp1p and the (◊◊◊◊) are the co-expression of sNlt1p and sSwp1p. In (d), (○○○○) are sNlt1p with tunicamycin, the (□□□□) are sSwp1p and (◊◊◊◊) are the co-expression of sNlt1p and sSwp1p with tunicamycin.

These data suggest that one of the three sets of proteins (sNlt1p/sSwp1p) is interacting and that this interaction results in a stabilization as observed by circular dichroism while the two remaining sets of proteins are not observed to be stabilizing each other.

A variety of studies have shown that carbohydrates play a critical role in mediating protein-protein interactions as well as in affecting the structure of proteins [28, 29]. The previously described observation that glycosylation affects the structure of sNlt1p but not its thermal stability hinted that the glycosylated state of sNlt1p might significantly influence the interaction between sNlt1p and sSwp1p. sNlt1p and sSwp1p were therefore co-expressed in the presence of tunicamycin in a roughly stoichiometric ratio as determined by SDS-PAGE and western blotting, purified and analyzed by circular dichroism thermal denaturation. Figure III-7d summarizes the result of this experiment. The melting temperature of sNlt1p expressed with tunicamycin is 62 C and the T_m of the co-expression of sNlt1p and sSwp1p in the presence of 1.0 μg/ml tunicamycin was determined to be 53 C, significantly less than that seen when sNlt1p was glycosylated. These data suggest that either the oligosaccharides on sNlt1p are interacting with sSwp1p or that the change in structure observed when sNlt1p is unglycosylated alters the interaction between this protein and sSwp1p.

In order to verify the interaction between sNlt1p and sSwp1p, the proteins were co-expressed, purified and loaded onto a Sephacryl S-200 column in order

to determine the elution profile of these proteins. The data from this experiment is shown below in Figure III-8.

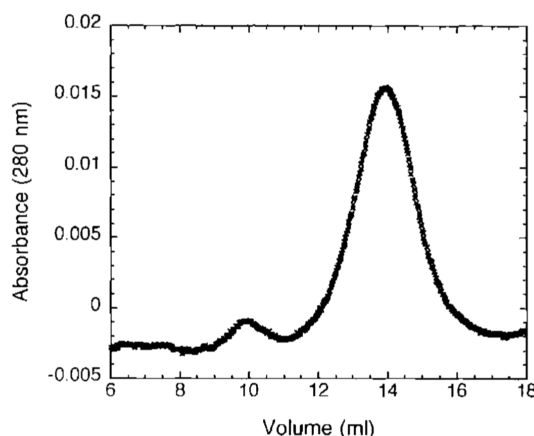


Figure III-8. Gel Filtration Trace of the co-expression of sNlt1p and sSwp1p.

The elution volume of the peak is 13.94 ml, which when compared to a standard curve corresponds to a molecular weight of 66 kDa. This mass is larger than that of sNlt1p and sSwp1p and suggests that these two proteins are interacting in solution when co-expressed.

III-6 Conclusions

Although the ultimate goal of this research is to reconstitute an active complex, the initial goals have been to identify a viable system where the proteins that make up the oligosaccharyl transferase enzyme complex can be expressed and purified in high yield. This chapter describes the initial steps towards this goal. Three soluble domains from the essential proteins are

monomeric, structured and can be purified in both high yield and purity. In addition, the two proteins with potential N-linked glycosylation sites, sNlt1p and sWbp1p, are heterogeneously glycosylated. Circular dichroism studies suggest that the proteins are structured and that the oligosaccharides on sNlt1p and sWbp1p mediate an important role in the quaternary structure of these proteins, further suggesting that these proteins need to be glycosylated in order to retain their native structure. An interaction between the soluble domains of sNlt1p and sSwp1p has been identified, implying that the luminal domains of these proteins are capable of interacting with each other. Finally, the oligosaccharides on sNlt1p either directly or indirectly mediate an interaction between sNtl1p and sSwp1p since the thermal stabilization observed between these proteins is abolished with the loss of glycosylation.

Combined, these results provide a framework for a further investigation into isolating an active oligosaccharyl transferase.

III-7 Acknowledgements

Thanks go to Peter Snow, Ph.D. from the Protein Expression Facility at the California Institute of Technology for introduction of the lytic baculovirus expression system and to Prof. Richard Murray and Christa Ziegler at the Department of Geology at Boston University for their ICP-ES expertise.

III-8 Experimental Section

Chemicals. Spodopteria frugiperda (Sf9) cells and Penicillin/Streptomycin were obtained from Invitrogen (Carlsbad, CA); the pAcGP67A expression vector and Baculogold was obtained from Pharmingen (San Diego, CA); fetal bovine serum was obtained from JRH Biosciences (Lenexa, KS); Sf-900 II SFM was purchased from Life Technologies (Rockville, MD); Ni-NTA resin was obtained from Qiagen (Valencia, CA); Tris Base was from Roche Diagnostics Corp. (Indianappolis, IN); Magnesium chloride was from Mallinckrodt (Hazelwood, MO); AEBSF, Leupeptin and Pepstatin A was purchased from Calbiochem (San Diego, CA); the Bicinchoninic acid (BCA) protein assay reagent kit, NBT/BCIP and goat anti-mouse secondary antibody were purchased from Pierce Biotechnology Co. (Rockford, IL); All other chemicals were purchased from Sigma.

Protein Construction/Expression/Purification. Soluble versions of Nlt1p, Swp1p and Wbp1p were expressed in a lytic baculovirus/insect cell expression system by using standard PCR techniques. The expression vectors pVL1393 from Invitrogen for Swp1p and Nlt1p and the pVT-Bac vector for Wbp1p were used for protein expression [30, 31]. Recombinant virus was generated by cotransfection of the transfer vector with linearized viral DNA, Baculogold. Recombinant virus was amplified and titered using Sf9 cells with a multiplicity of infection (M.O.I) of 0.3. Protein expression was initiated using cells at a density of $3x10^6$ cells/ml and a M.O.I. of 5. Cells were harvested three to four days post-infection. Protein was separated from supernatants of baculovirus-infected Sf9 cells using Ni-NTA chromatography, preceded by dialysis in 50 mM Tris, pH 7.5 and 2.5 mM $MgCl_2$. The protein inhibitors AEBSF (0.1 mM), Pepstatin A (0.5 μg/ml) and Leupeptin (0.5 μg/ml) were used throughout the purification. The cellular supernatant was loaded onto Ni-NTA resin in the presence of 15 mM imidazole, washed with 50 mM Tris, pH 7.5 and 2.5 mM $MgCl_2$ with 20 mM imidazole and eluted with 50 mM Tris, pH 7.5 and 2.5 mM $MgCl_2$ with 500 mM imidazole. Protein concentration was determined using the BCA Assay (Pierce).

SDS-PAGE was performed according to Laemmli [32]. Briefly, 15% polyacrylamide gels were poured and the electrophoresis was run at 100 V. The gels were stained with Brillant Blue R 250. To probe by western blot, the gels were electroblotted onto PVDF membrane at 100 V for one hour in Tris/Glycine transfer buffer with 20% methanol. The membrane was blocked in TBS (20 mM

Tris, pH 7.5 and 500 mM NaCl) with dry milk overnight. Following blocking, blots were sequentially blotted with mouse polyclonal antibodies specific to the protein of interest, goat anti-mouse secondary antibody conjugated to alkaline phosphatase and developed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP).

To probe the glycosylation of proteins, tunicamycin was added to the cell culture at a final concentration of 1.0 μg/ml at the initiation of viral infection. For N-terminal sequencing, protein was blotted on PVDF from Bio-Rad (Hercules, CA), detected with Ponceau S and submitted to the MIT Center for Cancer Research HHMI Biopolymers Laboratory.

Protein mass was determined using a PerSeptive Biosystems Mariner Biospectrometry Workstation using H_2O with 0.1% formic acid as solvent.

Gel Filtration Chromatography. A 200 μL aliquot of protein was passed through a 0.22 micron filter and loaded onto a Superdex 75 HR 10/30 pre-packed column on a Pharmacia Biotech fast protein liquid chromatography system (FPLC). Protein elution was monitored at 280 nm and the column buffer was 50 mM Tris, pH 7.5 and 2.5 mM magnesium chloride using a flow rate of 1 ml/min. Protein elution times were compared to a standard curve of ribonuclease A, chymotrypsinogen A, ovalalbumin and bovine serum albumin (Amersham Pharmacia). The void volume was determined using blue dextran 2000.

Analytical Ultracentrifugation. Protein was dialyzed for 72 hours in 50 mM sodium phosphate, pH 7.5 and 6 M guanidine-HCl or 50 mM sodium phosphate, pH 7.5, with 150 mM sodium chloride and 1 mM magnesium chloride at 4 C. Studies were performed on a Beckman Coulter Optima XL-I analytical ultracentrifuge. The experiment was run at 20 C. The molecular mass was determined using absorbance optics and the protein was quantitated using the BCA assay. Molecular masses were quantitated using WinNonlin V1.08 and Sednterp (Alliance Protein Laboratory).

Circular Dichroism. Protein samples were dialyzed for 24 hours in 50 mM sodium phosphate, pH 7.5 with or without 5 mM magnesium chloride. All CD measurements were performed on an Aviv Circular Dichroism Spectrometer Model 202 using strain free quartz cells with a pathlength of 0.1 mm. The molar elipticity at 222 nm was measured at 1 C intervals in triplicate as the temperature was raised using a bandwidth of 1 mm and an average time constant of 0.5 sec. The equilibrium time between measurements was 90 sec with a temperature dead band of 0.5 C. Measurements were taken from 4 C to 90 C. Blank spectra obtained over the same temperature range with buffer alone were subtracted. Full spectra were taken from 260-190 nm in triplicate at 4 C at 1 nm intervals.

Inductively coupled plasma-emission spectrometry. Protein was quantitated at 280 nm in 6 M guanidine-HCl and 10 mM sodium phosphate, pH 7.5, according to Pace et al [33]. Samples were injected in triplicate in a Jobin-Yvon Ultima C combined sequential and simultaneous ICP-ES and compared to a standard curve to determine metal concentration. Background metal concentrations were subtracted with blank injections.

III-9 References

1. Tierney, M. & Unwin, N. (2000) Electron microscopic evidence for the assembly of soluble pentameric extracellular domains of the nicotinic acetylcholine receptor, *J. Mol. Biol. 303*, 185-196.

2. McIntire, W., Myung, C., MacCleery, G., Wang, Q. & Garrison, J. (2002) Reconstitution of G protein-coupled receptors with recombinant G protein alpha and beta gamma subunits, *Methods Enzymol. 343*, 372-393.

3. Mikuni, O., Trager, J., Ackerly, H., Weinrich, S., Asai, A., Yamashita, Y., Mizukami, T. & Anazawa, H. (2002) Reconstitution of telomerase activity utilizing human catalytic subunit expressed in insect cells, *Biochem. Biophys. Res. Commun. 298*, 144-150.

4. Bozon, W., Couture, L., Pajot-Augy, E., Richard, F., Remy, J. & Salesse, R. (2002) Rescue of intracellularly trapped lutropin receptor exodomain by

endodomain and reconstitution of a functional membrane receptor: Interaction between exo- and endodomains, *Protein Expres. Purif. 25*, 114-123.

5. Nemeth, J., Hochensang, G., Marnett, L. & Caprioli, R. (2001) Characterization of the glycosylation sites in cyclooxygenase-2 using mass spectrommetry, *Biochemistry. 40*, 3109-3116.

6. Pathak, R., Parker, C. & Imperiali, B. (1995) The essential yeast NLT1 gene encodes the 64 kDa glycoprotein subunit of the oligosaccharyl transferase, *FEBS Lett. 362*, 229-234.

7. te Hessen, S., Knauer, R., Lehle, L. & Aebi, M. (1993) Yeast Wbp1p and Swp1p form a protein complex essential for oligosaccharyl transferase activity, *EMBO J. 12*, 279-284.

8. te Hessen, S., Rauhut, R., Aebersold, R., Abelson, J., Aebi, M. & Clark, M. (1991) An essential 45 kDa yeast transmembrane protein reacts with anti-nuclear pore antibodies: purification of the protein, immunolocalization and cloning of the gene, *Eur. J. Cell Biol. 56*, 8-18.

9. Heifetz, A., Keenan, R. & Elbein, A. (1979) Mechanism of action of tunicamycin on the UDP-GlcNAc-dolichyl-phosphate GlcNAc-1-phosphate transferase, *Biochemistry. 18*, 2186-2192.

10. Silberstein, S., Collins, P., Kelleher, D. & Gilmore, R. (1995) The essential Ost2 gene encodes the 16-KD subunit of the yeast oligosaccharyltransferase, a highly conserved protein expressed in diverse eukaryotic organisms, *J. Cell Biol. 131*, 371-383.

11. Karaoglu, D., Kelleher, D. & Gilmore, R. (1997) The highly conserved Stt3p protein is a subunit of the yeast oligosaccharyltransferase and forms a subcomplex with Ost3p and Ost4p, *J. Biol. Chem. 272*, 32513-32520.

12. Spirig, U., Glaras, M., Bodmer, D., Reiss, G., Burda, P., Lippuner, V., te Hessen, S. & Aebi, M. (1997) The Stt3 protein is a component of the yeast oligosaccharyltransferase complex, *Mol. Gen. Genet. 256*, 628-637.

13. Zufferey, R., Knauer, R., Burda, P., Stagljar, I., te Hessen, S., Lehle, L. & Aebi, M. (1995) Stt3, a highly conserved protein required for yeast oligosaccharyl transferase activity in-vivo, *EMBO J. 14*, 4949-4960.

14. Johnson, M., Correia, J., Yphantis, D. & Halvorson, H. (1981) Analysis of data from the analytical ultra-centrifuge by non-linear least-squares techniques, *Biophys. J. 36*, 575-588.

15. Hinz, H. (1986) *Thermodynamic data for biochemistry and biotechnology*, Springer-Verlag, New York.

16. Glasel, J. & Deutscher, M. (1995) *Introduction to biophysical methods for protein and nucleic acid research*, Academic Press, San Diego.

17. Armstrong, J. & McKenzie, H. (2001) Apparently Anomalous Sedimentation Behavior in MIxed Solvent Systems with Strong Interactions between Solution Components: Analysis of Nonideal Behavior by Bovine Serum Albumin in 7 M Urea at pH 3.3, *J. Prot. Chem. 20*, 255-263.

18. Hendrickson, T. & Imperiali, B. (1995) Metal-ion dependence of oligosaccharyl transferase-implications for catalysis, *Biochemistry. 34*, 9444-9450.

19. Poussel, E. & Mermet, J. (1993) Simple experiments for the control, the evaluation and the diagnosis of inductively coupled plasma sequential systems, *Spectrochimica Acta. 48B*, 743-755.

20. Jarvis, I. & Jarvis, K. (1992) Plasma spectrometry in the earth sciences: techniques, applications, and future trends, *Chem. Geol. 95*, 1-33.

21. Pathak, R., Hendrickson, T. & Imperiali, B. (1995) Sulfhydryl modification of the yeast Wbp1p inhibits oligosaccharyl transferase activity, *Biochemistry. 34*, 4179-4185.

22. Merelo, J., Andrade, M., Prieto, A. & Moran, F. (1994) Proteinotopic feature maps, *Neurocomputing. 6*, 443-454.

23. Andrade, M., Chacon, P., Merelo, J. & Moran, F. (1993) Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network, *Prot. Eng. 6*, 383-390.

24. Yan, Q. & Lennarz, W. (1999) Oligosaccharyltransferase: A complex multisubunit enzyme of the endoplasmic reticulum, *Biochem. Biophys. Res. Commun. 266*, 684-689.

25. Stagljar, I., Korostensky, C., Johnsson, N. & te Hessen, S. (1998) A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo, *Proc. Natl. Acad. Sci. U.S.A. 95*, 5187-5192.

26. Fu, J., Ren, M. & Kreibach, G. (1997) Interactions among subunits of the oligosaccharyltransferase complex, *J. Biol. Chem. 272*, 29687-29692.

27. Wills, F., McCubbin, W. & Kay, C. (1994) Smooth-muscle calponin-caltropin interaction-Effect of biological activity and stability of calponin, *Biochemistry. 33*, 5562-5569.

28. Rizzuto, C., Wyatt, R., Hernandez-Ramos, N., Sun, Y., Kwong, P., Hendrickson, W. & Sodroski, J. (1998) A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding, *Science. 280*, 1949-1953.

29. O'Connor, S., Pohlmann, J., Imperiali, B., Saskiawan, I. & Yamamoto, K. (2001) Probing the effect of the outer saccharide residues of N-linked glycans on peptide conformation, *JACS. 123*, 6187-6188.

30. Tessier, D., Thomas, D., Khouri, H., Laliberte, F. & Vernet, T. (1991) Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide, *Gene. 98*, 177-183.

31. Luckow, V. & Summers, M. (1988) Trends in the development of baculovirus expression vectors, *Bio/Technology. 6*, 47-55.

32. Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature. 227*, 680-685.

33. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. & Gray, T. (1996) How to measure and predict the molar absorption coefficient of a protein, *Protein Sci. 4*, 2411-2423.

Chapter IV: Summary and Future Directions

IV-1 Summary of Dissertation

The studies within this dissertation have included the cloning, expression and purification of four essential subunits of the yeast *S. cerevisiae* oligosaccharyl transferase. These proteins, although present as large insoluble aggregates in *P. pastoris* were sufficiently solubilized in the lytic baculovirus expression system by non-ionic detergents to initiate reconstitution studies. Both Nlt1p and Wbp1p, which encode N-linked glycosylation sites, were determined to be glycosylated by western blot analysis following the addition of tunicamycin to baculovirus-infected insect cells.

The primary obstacle in the reconstitution experiments was retention of catalytic activity from the insect cells which was retained during initial purification attempts. A variety of methods, including gel filtration and heparin chromatography, as well as the use of reducing agents eliminated endogenous catalysis. Reconstitution of these four proteins proved unsuccessful when the subunits were expressed and purified independently and then combined in liposomes and as a detergent-solubilized solution. In addition, co-expression of the four proteins also was not sufficient for catalytic competency. Reconstitution of catalytic activity was only successful with the purification and analysis of Stt3p. This subunit was originally thought to be a substoichiometric assembly factor, but

recent data has suggested that this protein is critical for catalysis [1, 2]. The data within this dissertation suggests that the four essential subunits, Nlt1p, Ost2p, Swp1 and Wbp1p are not sufficient for catalysis under these experimental conditions and that Stt3p is required for a competent enzyme.

In order to simplify the complexity of the yeast oligosaccharyl transferase, the soluble domains of three essential subunits from the yeast oligosaccharyl transferase were cloned. Three constructs were initially expressed in *P. pastoris*, although the proteins were present primarily as insoluble aggregates. The proteins were expressed in the lytic baculovirus expression system to take advantage of the co- and post-translational modifications inherent in this expression system. A characterization of three essential soluble domains of the oligosaccharyl transferase revealed that the proteins are monomeric in solution and structured. Both proteins with N-linked glycosylation sequences were glycosylated as determined by western blot analysis. The proteins, sNlt1p and sSwp1p interacted with each other as determined by circular dichroism and gel filtration chromatography. This association was mediated by the glycosylation state of sNlt1p.

Collectively, the data presented provides evidence that the four proteins Nlt1p, Ost2p, Swp1p and Wbp1p are not sufficient for activity and that Stt3p is required for catalysis. In addition, glycosylation is critical to the secondary structure of these proteins, further suggesting that reconstitution of catalysis for oligosaccharyl transferase will be optimized by the expression of the glycosylated

proteins in organisms which are capable of co-translational modifications. The soluble constructs are well-behaved and can be further analyzed in order to examine the affinity of sNlt1p and sSwp1p using techniques such as analytical ultracentrifugation.

IV-2 Future Directions

This is an exciting time for reconstitution of the essential subunits of oligosaccharyl transferase. The next step in understanding the mechanism of catalysis for the enzyme is to clone and purify the final essential subunit, Stt3p. Initial experiments in the lab have cloned the gene from *S. cerevisiae* and research is now focused on expressing this construct as a fusion protein due to the high hydrophobicity of this subunit. Characterization of this protein will culminate in elucidating whether Stt3p is sufficient for catalytic competency of the yeast oligosaccharyl transferase. In order to verify the importance of this subunit, mutagenesis of this protein within highly conserved regions will be able to elucidate whether this proteins is sufficient for catalysis. Throughout the study of the enzyme, this subunit has proven a difficult subject of study. As a protein with twelve predicted transmembrane domains, the subunit will naturally be expressed at low levels in cells. The protein does not stain well with Coomassie Blue or silver stain and on SDS-PAGE, the protein runs significantly smaller (60 kDa) than the mass predicted by primary sequence analysis (78 kDa) [3]. This further obscures the identification of this protein in enzyme preparations.

However, construction of a fusion protein with encodes a protein with a sensitive antibody should minimize the difficulty in identification of this protein

In order to examine the importance of this subunit without large-scale protein purification, an interesting *in vivo* experiment could take advantage of the fact that Stt3p is homologous over species. In this experiment, a mutant of Stt3p, known to be lethal to yeast, could be stably transfected into insect cells and the level of glycosylation on a marker protein within the cells could be measured against untransfected cells [2]. If this protein is interacting with the native glycosylation machinery, one would expect a decreased rate of glycosylation for a marker protein, such as carboxypeptidase, since the overexpressed mutant Stt3p would replace the endogenous Stt3p homolog from the *Sf9* cells and reduce glycosylation efficiency.

In order to further examine the structure of these proteins, x-ray crystallography could be utilized to probe the structure of the soluble domains of the yeast oligosaccharyl transferase. Preliminary electron microscopy data by Prof. Chris Akey at the Boston University School of Medicine has elucidated a low-resolution structure of the OT complex from yeast (personal communication). However, it is difficult to orient the molecule relative to the lipid bilayer or to identify individual subunits from this complex because the structure lacks any orientation. An interesting method by which to model the subunits into this complex would be to obtain crystal structures of the soluble domains and dock them into the structure obtained by Akey and co-workers, providing direct

evidence to how these subunits interact with each other. It would also be interesting to determine whether the soluble domain of Stt3p is sufficient for catalysis or whether the subunit interacts with the three other key essential soluble domains. One complication to this experiment is that the highly conserved consensus sequence is directly adjacent to a proposed transmembrane domain [1, 2, 4]. However, multiple constructs could be expressed to determine the optimal size of the subunit.

IV-3 References

1. Wackler, M., Linton, D., Hitchen, P., Nita-Lazar, M., Haslam, S., North, S., Panico, M., Morris, H., Dell, A., Wren, B. & Aebi, M. (2002) N-linked protein glycosylation in Campylobacter jejuni and its functional transfer into E. coli, *Science. 298*, 1790-1793.

2. Yan, Q. & Lennarz, W. (2002) Studies on the function of oligosaccharyl transferase subunits: Stt3p is directly involved in the glycosylation process, *J. Biol. Chem. 277*, 47692-47700.

3. Karaoglu, D., Kelleher, D. & Gilmore, R. (1997) The highly conserved Stt3p protein is a subunit of the yeast oligosaccharyltransferase and forms a subcomplex with Ost3p and Ost4p, *J. Biol. Chem. 272*, 32513-32520.

4. Zufferey, R., Knauer, R., Burda, P., Stagljar, I., te Hessen, S., Lehle, L. & Aebi, M. (1995) Stt3, a highly conserved protein required for yeast oligosaccharyl transferase activity in-vivo, *EMBO J. 14*, 4949-4960.

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PUBLICATIONS

1. Dempski, R.E. and Imperiali, B. Stability of the essential soluble domains of oligosaccharyl transferase-Effect of glycosylation on protein-protein interactions. Submitted.
2. Dempski, R.E. and Imperiali, B. Oligosaccharyl Transferase: Gatekeeper to the secretory pathway. Curr. Opin. Chem. Biol., 6, 844-850 (2002).
3. Xia, M., Dempski, R., and Hille, R. The reductive half-reaction of xanthine oxidase-Reaction with aldehyde substrates and identification of the catalytically labile oxygen. J. Biol. Chem., 274, 3323-3330 (1999).
4. Xia, M., Ilich, P., Dempski, R., and Hille, R. Recent mechanistic studies of xanthine oxidase. Biochem. Soc. Trans., 25, 768-773 (1997).
5. Olsen, D.B., Benseler, F., Cole, J.L., Stahlhut, M.W., Dempski, R.E., Darke, P.L., and Kuo, L.C. Elucidation of basic mechanistic and kinetic properties of influenza endonuclease using chemically synthesized RNAs. J. Biol. Chem., 271, 7435-7439 (1996).

PRESENTATIONS

1. Investigation of the *Saccharomyces cerevisiae* oligosaccharyl transferase. Dempski, R.E., Glover, K.J., and Imperiali, B. Boston ACS meeting 2002, Abstracts of Papers of the American Chemical Society 224: 057-Biol Part 1 Aug 18 2002.
2. Recent mechanistic studies of xanthine oxidase. Xia, M., Ilich, P., Dempski, R., and Hille, R. Meeting of the Biochemical Society, Bath England, April 1997.