Sulfhydryl Modification of the Yeast Wbp1p Inhibits Oligosaccharyl Transferase Activity[†,‡]

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ABSTRACT: Chemical labeling of the multimeric *Saccharomyces cerevisiae* oligosaccharyl transferase indicates that the 48 kDa Wbp1p subunit is an integral component of the catalytically active enzyme. The enzyme was purified following chromatography on concanavalin A agarose, heparin agarose, Q-Sepharose, and hydroxyapatite media. The enzyme activity copurified with a tetrameric complex of polypeptide subunits. Two of the subunits have been identified as the yeast proteins Wbp1p and Swp1p by amino-terminal residue sequencing. A third subunit was identified as a variably glycosylated polypeptide near 64 kDa; preliminary amino acid sequencing showed no identity to known yeast proteins. Modification of a cysteine residue by the reagent methyl methanethiolsulfonate (MMTS) caused time-dependent and concentration-dependent inactivation of the enzyme. To identify the modified subunit of the transferase complex, the labeling reagent *S*-[(*N*-biotinoylamino)ethyl] methanethiolsulfonate (BMTS) was synthesized. Like MMTS, BMTS inactivated the oligosaccharyl transferase in a time-dependent manner. Additionally, incubation with the substrate (dolichylpyrophosphoryl)-*N*,*N*′-diacetylchitobiose [Dol-PP(GlcNAc)$_2$] protected the enzyme from BMTS inactivation. When the purified enzyme complex was incubated with BMTS, Wbp1p alone was specifically labeled, thereby associating this subunit with catalysis and the binding of the dolichylpyrophosphoryl oligosaccharide substrate in the transferase reaction.

In eukaryotic cells, most secreted, vesicular, and integral membrane proteins bear covalently linked carbohydrate groups on asparagine, serine, or threonine residues. The oligosaccharide chains of glycoproteins contribute to their conformation and stability and also participate in cellular targeting and recognition processes (Herscovic & Orlean, 1993). In catalyzing the *N*-linked glycosylation of proteins, oligosaccharyl transferase acts at the nexus of two essential biochemical pathways. The enzyme brings together a complex lipid-linked oligosaccharide donor, dolichylpyrophosphoryl-GlcNAc$_2$Man$_9$Glc$_3$,[1] which is synthesized by a series of glycosyl transferases in the membrane of the rough endoplasmic reticulum, and an acceptor peptide sequence which is part of a nascent polypeptide chain being translocated into the lumen of the rough endoplasmic reticulum (Herscovics & Orlean, 1993; Kukurzinska et al., 1987; Tanner & Lehle, 1987). Specifically, the peptide acceptor reacts at the side chain of asparagine residues in selected Asn-X-Thr/Ser sequences where X is any residue other than proline (Marshall, 1974). Since the features of both acceptor and donor in the oligosaccharyl transferase reaction are identical in yeast and higher eukaryotes including the mammalian species, the accessible genetics of *Saccharomyces cerevisiae* provide an ideal system for the study of the enzyme.

The molecular characterization of the yeast oligosaccharyl transferase has associated a heteromeric membrane complex with enzymatic activity. The yeast proteins Wbp1p and Swp1p are both necessary for *in vivo* transferase activity; when the *WBP1* gene or the *SWP1* gene was placed under the control of the repressible *GAL* promoter, the depletion of either gene product correlated with a loss of enzyme activity (te Heesen et al., 1992, 1993). However, simultaneous overexpression of both gene products did not yield a significant increase in transferase activity (te Heesen et al., 1993). Two independent purifications of the enzyme from yeast membranes have yielded complexes of four and six polypeptides (Knauer & Lehle, 1994; Kelleher & Gilmore, 1994); both complexes include the Wbp1p and Swp1p proteins as well as several novel proteins. Based on the amino acid sequences of the two characterized subunits, the yeast enzyme complex shows significant homology to the oligosaccharyl transferase complexes purified from higher eukaryotes (Kelleher et al., 1992; Kumar et al., 1994). The canine pancreas enzyme was purified as a trimeric membrane protein complex including ribophorin I, ribophorin II, and OST48 (Kelleher et al., 1992; Silberstein et al., 1993). Wbp1p is homologous to the OST48 subunit (Silberstein et al., 1992), and Swp1p is homologous to the carboxy-terminal half of the ribophorin II subunit (Kelleher & Gilmore, 1994); presumably, the amino acid sequence of one of the other protein subunits of the yeast enzyme will show homology to ribophorin I. Comprehensive and accurate study of the

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[1] Abbreviations: GlcNAc, *N*-acetylglucosamine; Man, mannose; Glc, glucose; MMTS, methyl methanethiolsulfonate; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; α-MeMan, α-methylmannopyranoside; Dol-PP, dolichol pyrophosphate; IAA, iodoacetic acid; IAM, iodoacetamide; NEM, *N*-ethylmaleimide; BMTS, *S*-[(*N*-biotinoylamino)ethyl] methanethiolsulfonate; DTT, dithiothreitol; dpm, disintegrations/min; PC, phosphatidylcholine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NP-40, Nonidet P-40.

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enzyme requires the identification of all the subunits essential for oligosaccharyl transferase activity.

To begin the examination of the chemical roles of each subunit of the yeast enzyme complex, we have developed an independent purification of the oligosaccharyl transferase. Enzymatic activity copurified with a tetrameric complex. Two of the polypeptide subunits were identified by amino-terminal residue sequencing; one is the 48 kDa protein Wbp1p (te Heesen et al., 1992), and the other is the 32 kDa protein Swp1p (te Heesen et al., 1993). In addition, a novel 64 kDa glycoprotein and a nonglycosylated 34 kDa polypeptide also cofractionated with activity. We examined the purified enzyme complex with a series of chemical modification reagents and show here that oligosaccharyl transferase activity is sensitive to inactivation by the sulfhydryl-directed reagent MMTS (Smith et al., 1975). To visualize the modification of the enzyme, we have developed and synthesized a biotinylated version of the alkyl alkanethiolsulfonate and now demonstrate that a single component of the purified enzyme, the Wbp1p protein, is labeled by this novel reagent.

Our experiments provide the first direct chemical evidence associating the Wbp1p protein with oligosaccharyl transferase catalytic activity. These results will stimulate future experiments on understanding the role of the chemically modified residue in transferase catalysis.

MATERIALS AND METHODS

Oligosaccharyl Transferase Assay. Oligosaccharyl transferase activity was measured as described (Imperiali & Shannon, 1991) with the following modifications. For each assay, 440 pmol of [^{3}H](dolichylpyrophosphoryl)-*N*,*N*'-diacetylchitobiose (0.22 nCi/pmol) (Imperiali & Zimmermann, 1990) in 3:2 $CHCl_3$:MeOH was evaporated under nitrogen and redissolved in 10 μL of DMSO containing 2.5 mM Bz-Asn-Leu-Thr-NHMe. At the start of each assay, an aliquot of the experimental sample, typically 10−20 μL, was mixed with the peptide and lipid disaccharide substrates and incubated for 10 min in a final volume of 200 μL of 2.2 μM Dol-PP-GlcNAc-[^{3}H]GlcNAc, 125 μM Bz-Asn-Leu-Thr-NHMe, 140 mM sucrose, 50 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1.0% Nonidet P-40, and 0.5 mg/mL PC. Both substrates were present in the assays near their experimentally determined K_m concentrations. At each of four different time points spanning the 10 min incubation, a 40 μL aliquot was removed from the reaction mixture and quenched into 1.2 mL of 3:2:1 $CHCl_3$:MeOH:4 mM $MgCl_2$. Aliquots of each assay reaction were measured at four different time points to ensure accurate and linear kinetic measurement of the amount of enzyme present in each sample. The aqueous layer was separated, and the organic layer was extracted twice with 0.6 mL of 192:186:12:2.69 MeOH:H_2O:$CHCl_3$:250 mM $MgCl_2$. The combined aqueous extracts were counted in EcoLite(+) (ICN) on a Beckman LS-5000TD scintillation counter. After the organic layer had evaporated, the proteinaceous residue was dissolved in 200 μL of Solvable (New England Nuclear) and counted in Formula 989 (New England Nuclear). The amount of radioactivity transferred into the aqueous extracts was normalized against the total radioactivity present in the aqueous extracts and the organic layer. Oligosaccharyl transferase activity was measured as the rate of transfer of the labeled disaccharide from the dolichylpyrophosphoryl carrier to the acceptor peptide; activity was expressed as pmol of peptide glycosylated/min.

Buffers. Buffer A contained 50 mM Tris-Cl, pH 7.5, and 2.5 mM $MgCl_2$. Buffer B contained 140 mM sucrose, 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.1 mM AEBSF (CalBiochem), 0.5 μg/mL pepstatin A, and 0.5 μg/mL leupeptin. Buffer C contained 140 mM sucrose, 50 mM HEPES, pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 0.4% Nonidet P-40, 0.5 mg/mL PC, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. Buffer D contained 140 mM sucrose, 50 mM HEPES, pH 7.5, 25 mM NaCl, 10 mM $MgCl_2$, 0.4% Nonidet P-40, and 0.5 mg/mL PC. Buffer E contained 140 mM sucrose, 20 mM MES, pH 6.4, 25 mM NaCl, 10 mM $MgCl_2$, 0.4% Nonidet P-40, and 0.1 mg/mL PC. Buffer F contained 140 mM sucrose, 50 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 0.4% Nonidet P-40, and 0.1 mg/mL PC. Buffer G contained 140 mM sucrose, 50 mM HEPES, pH 7.5, 0.6% Nonidet P-40, and 0.5 mg/mL PC.

Purification. Yeast strain PRY46 (*MAT*α *his- pep4-3 prc1-1126 prb1-1122*; P. Robbins, personal communication) was grown to an OD of approximately 1.5 in 240 L of YPD media. The cells were frozen at −80 °C. Cells were thawed in 100 g batches overnight to 4 °C and then washed twice with buffer A. All further purification steps were carried out at 4 °C. The cells were suspended to a total volume of 150 mL in buffer A plus protease inhibitors (0.1 mM AEBSF, 0.5 μg/mL pepstatin A, 0.5 μg/mL leupeptin; CalBiochem) and beaten for 16 20 s intervals with 40 s cooling intervals in a glass bead beater (BioSpec) with 100 mL of 425−600 μm glass beads (Sigma). The suspension was harvested, and the beads were washed with a total of 50 mL of buffer A; the suspension and the washes were combined and spun at 8 K for 10 min. The supernatant was saved, and the pellets were resuspended in 100 mL of buffer A and again spun at 8 K for 10 min; the supernatants were combined and spun at 8 K for 10 min. Microsomal membranes were pelleted from this supernatant by centrifugation at 40 K for 60 min in a Beckman 45Ti rotor. The membranes were suspended at an A_{280} of 50 in 60 mL of buffer B. This suspension was brought to 0.5 M NaCl and incubated for 15 min (Walter & Blobel, 1983); the membranes were recovered after centrifugation at 40 K for 60 min. The pellet was suspended in 60 mL of buffer B with 0.1 M NaCl and 0.05% Nonidet P-40 (Sigma) and incubated for 15 min; the membranes were recovered by centrifugation at 40 K for 60 min. Membrane proteins were partially solubilized in 68 mL of buffer B with 0.6% Nonidet P-40 and 0.5 M NaCl, incubated for 15 min, and clarified at 40 K for 60 min.

Concanavalin A agarose (10 mL; Vector) was equilibrated by washing twice with 30 mL of buffer C. The solubilized membrane fraction (68 mL) was incubated with the media overnight at 4 °C. The medium was recovered by centrifugation at 1 K and washed three times with 30 mL of buffer C. Bound proteins were eluted by three successive 4 h incubations with 70 mL of 750 mM α-MeMan in buffer C. The eluate (200 mL) was dialyzed overnight against 140 mM sucrose, 17 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.4% Nonidet P-40, and 0.1 mg/mL PC to dilute the α-MeMan.

A 2.5 mL heparin agarose (Pierce) column was equilibrated in buffer D, and the pooled concanavalin A eluate was applied to the column at a flow rate of 1.0 mL/min. Activity was eluted sharply with a 50 mL gradient of 25−

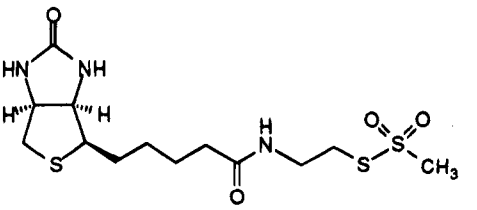
500 mM NaCl in buffer D at a flow rate of 0.5 mL/min. The active fractions (20 mL) were dialyzed overnight with one change against 500 mL of buffer E.

A 2.5 mL Q-Sepharose (Pharmacia) column was equilibrated in buffer E plus 0.5 mg/mL PC. Twenty milliliters of the heparin eluate was applied at a flow rate of 0.5 mL/min. Activity was eluted with a gradient of 25−200 mM NaCl in buffer E plus 0.5 mg/mL PC. The active fractions (20 mL) were applied directly to a 1.0 mL BioGel HTP hydroxyapatite (BioRad) column. The column was washed with 1.0 M NaCl in buffer E plus 0.5 mg/mL PC, and the enzyme was eluted with a gradient of 0−0.8 M NaF in buffer E plus 0.5 mg/mL PC. A total of 12 mL of the active fractions was dialyzed overnight with one change against 500 mL of buffer F.

Analytical Techniques. Purified protein was precipitated with chloroform and methanol (Wessel & Flügge, 1984) prior to separation by SDS−PAGE (Laemmli, 1970). Separated proteins were stained with Coomassie brilliant blue R250. For immunological detection of Wbp1p, separated proteins were transferred to nitrocellulose (Towbin et al., 1979) and then incubated in 1:1000 diluted antiserum raised against Wbp1p (te Heesen et al., 1991) followed by a 1:1000 diluted anti-rabbit IgG alkaline phosphatase conjugate (ICN). For the detection of glycoproteins, nitrocellulose-bound polypeptides were incubated in 10 μg/mL biotinylated concanavalin A (Sigma) followed by an avidin-biotinylated alkaline phosphatase complex (Pierce). Glycoproteins were deglycosylated with PNGaseF (New England Biolabs) in 1.0% β-mercaptoethanol, 1.0% Nonidet P-40, 0.5% SDS, and 50 mM sodium phosphate, pH 7.5. For amino acid sequencing, protein bands were transferred to ProBlott (Applied Biosystems) and sequenced by the California Institute of Technology Protein and Peptide Micro Analytical Facility. The total protein in each purification fraction was determined by the Micro BCA assay (Pierce) after precipitation with chloroform and methanol.

S-[(N-Biotinoylamino)ethyl] Methanethiolsulfonate (BMTS). (A) Synthesis. *S*-(Aminoethyl) methanethiolsulfonate hydrobromide (44 mg, 0.19 mmol) (Bruice & Kenyon, 1982) and (+)-biotin *p*-nitrophenyl ester (70 mg, 0.19 mmol) were dissolved in 0.4 mL of anhydrous dimethylformamide and cooled to 0 °C. Triethylamine (24 μL, 0.19 mmol) was then added dropwise with stirring. After 2 h at this temperature an additional portion of *S*-(aminoethyl) methanethiolsulfonate hydrobromide (10 mg, 0.05 mmol) was added. The reaction mixture was then stirred for 2 h at which time the solvent was removed under low pressure at room temperature. The crude residue was then redissolved in a minimal volume of warm methanol, filtered, and cooled to 0 °C. The coupled product crystallized readily from the filtrate. BMTS was further purified by two additional recrystallizations from methanol. The reaction afforded 50 mg (70% yield) of pure white crystalline product.

(B) Physical Data. Mp: 108−109 °C. MS: [MH^+] calcd for $C_{13}H_{23}N_3O_4S_3$, 382; obsd, 382. ^{1}H NMR (DMF-d_7): δ 1.36−1.76 (m, 6H), 2.19 (t, 2H, J = 7.4 Hz), 2.82 (AB, 2H, J = 0, 4.9, and 12.4 Hz), 3.18 (m, 1H), 3.37 (t, 1H, J = 6.3 Hz), 3.52 (t, 1H, J = 6.0 Hz), 3.58 (s, 3H), 4.28 (m, 1H), 4.45 (m, 1H), 6.38 (s, 1H), 6.47 (s, 1H), 8.14 (t, 1H, J = 5.2 Hz). ^{13}C NMR (DMF-d_7): δ 26.1, 29.0, 29.1, 36.0, 36.3, 39.1, 40.7, 50.6, 56.4, 60.5, 62.1, 163.7, 173.5.



Chemical Inactivation of Oligosaccharyl Transferase. Modification of the yeast oligosaccharyl transferase with MMTS was performed in buffer G at 25 °C in the presence of 10 mM $MnCl_2$ at a protein concentration of 23.6 μg/mL. MMTs was added to 114 μL of pure enzyme solution in 6 μL of methanol to yield final reagent concentrations of 0, 0.5, 1.0, 2.0, and 4.0 mM. At various time intervals after MMTS addition, 30 μL of the preincubation mixture was diluted to 200 μL into the transferase assay buffer containing both peptide and lipid-linked oligosaccharide substrates. The loss of catalytic activity was monitored over 40 min. In the absence of MMTS, the enzyme maintained full activity over this time period. Similar experiments in the presence of 10 mM IAA, IAM, and NEM did not significantly affect enzymatic activity.

Approximately 50% of the activity was recovered by incubation with 40 mM DTT after 4 days at 4 °C. Unmodified oligosaccharyl transferase activity was not affected by the addition of DTT. Attempts to completely regenerate enzymatic activity with tributylphosphine (Nishimura et al., 1975) were hindered due to the lability of the enzyme in the presence of this reagent at a 1 mM concentration.

BMTS inactivation was carried out as described for MMTS at a final concentration of 2.5 mM. Inactivation was also performed in the presence of Dol-PP(GlcNAc)$_2$ (Imperiali & Zimmerman, 1990) in order to demonstrate substrate protection. For this experiment, 100 μL of the enzyme was incubated for 10 min with 1 × 10^6 dpm 4.4 μM [^{3}H]Dol-PP(GlcNAc)$_2$ (specific activity 0.22 Ci/mmol). After this time BMTS (2.5 mM) was added. At 15 min, 20 μL of the incubation mixture was assayed for transferase activity in the assay buffer containing peptide substrate. For the control experiments, the Dol-PP(GlcNAc)$_2$ in the incubation mixture was omitted and an equivalent amount of this substrate was added to the assay buffer. Chitobiose and dolichol phosphate were also examined for substrate protection by preincubating the enzyme with 250 μM of each compound before the addition of MMTS.

To visualize the BMTS-treated protein, 300 μL of pure protein solution in buffer G at 25 °C in the presence of 10 mM $MnCl_2$ was treated with 0.5 mM MMTS for 10 min to block any alkylation sensitive sites. The reaction was then quenched with an excess of cysteine and the mixture dialyzed against buffer G (2 × 50 mL). The dialysate was then harvested and adjusted to 10 mM $MnCl_2$ and 2.5 mM BMTS. At time intervals (0.5, 2.0, 5.0, and 30.0 min), 50 μL aliquots were withdrawn from the incubation mixture and brought to 50 mM cysteine. Two 50 μL aliquots were quenched at the 30 min time point. The protein was then precipitated with chloroform and methanol (Wessel & Flügge, 1984). The samples collected at the four time intervals were then redissolved in 125 μL of nonreducing sample buffer (62.5 mM Tris-Cl, pH 6.8, 10% glycerol, 2% SDS, and 0.025% bromophenol blue), while the additional 30 min time point sample was redissolved in the same volume of sample buffer

Table 1: Purification of the Yeast Oligosaccharyl Transferase[a]

fraction	total activity (pmol/min)	total protein (mg)	specific activity (pmol/min/mg)	enrichment (-fold)
yeast microsomes	(2960)	756	3.9	1.0
NP-40 solubilization	2960	150	19.7	5.1
concanavalin A eluate	1020	5.6	182	46.7
heparin agarose eluate	490	1.6	306	78.5
Q-Sepharose eluate	350	0.22	1590	408

[a] Fractions from the purification of the enzyme were assayed for total transferase activity and total protein according to the experimental. The total activity of the microsomal fraction is approximate due to the instability of the enzyme in a microsomal suspension. The enzyme could be further purified by hydroxyapatite with a 2-fold increase in enrichment.

with 40 mM DTT. Samples for electrophoresis were incubated at 90 °C for 10 min. Ten microliters of each sample was loaded onto two 12% polyacrylamide gels for electrophoresis and transfer to nitrocellulose. One membrane was probed for biotin-labeled proteins with the avidin-biotinylated alkaline phosphatase complex (Pierce); the other was treated with anti-Wbp1p antiserum (te Heesen et al., 1991).

RESULTS

Purification. The yeast oligosaccharyl transferase was partially purified from a crude microsomal membrane extract (see Table 1). The purification was followed by the assay described above which monitors the transfer of a labeled disaccharide from a dolichylpyrophosphate donor to an acceptor Bz-Asn-Leu-Thr-NHMe tripeptide; both donor and acceptor substrates were present in the assays at concentrations comparable to their experimentally determined K_m concentrations. Yeast cells were lysed, and the microsomal membrane fraction was isolated and suspended in buffer; the activity of this initial microsomal fraction was estimated because of the instability of the enzyme in membrane suspensions (see Table 1). The membranes were washed with NaCl to remove peripherally associated proteins and then with detergent at a low NaCl concentration to deplete luminal proteins and partially solubilize the integral membrane protein fraction. The enzyme was solubilized with the detergent Nonidet P-40 at up to a 90% yield of the estimated activity in the membrane fraction; the solubilized enzyme remained active for weeks at 4 °C. Enzyme activity was enhanced with PC (Chalifour & Spiro, 1988); in addition, the phospholipid increased the stability of the detergent-solubilized enzyme, and chromatography experiments did not yield active enzyme when it was omitted from the medium.

The solubilized extract was applied to a concanavalin A affinity column to which the enzyme bound tightly. Efficient elution of the activity required a high concentration of the lectin inhibitor α-MeMan and up to 20 media volumes of elution buffer over 12 h. After dilution of the lectin inhibitor by dialysis, the enzyme was bound to heparin agarose and eluted with an NaCl gradient. Enzyme activity was then bound to Q-Sepharose at a slightly lowered pH and eluted with a gentle NaCl gradient. The enzyme could be further purified with hydroxyapatite media with a NaF gradient elution; active enzyme could not be eluted from this media

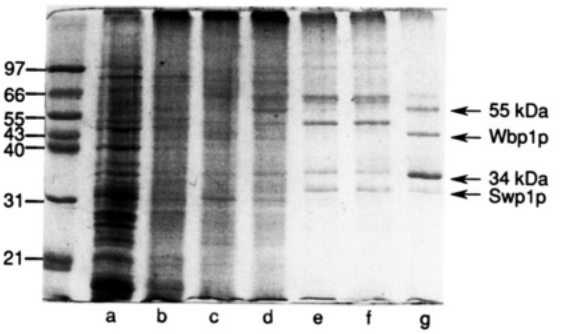


FIGURE 1: Purification of the yeast oligosaccharyl transferase. The yeast oligosaccharyl transferase was purified according to the protocol outlined in the experimental. Samples of each purification fraction were separated by SDS−polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue R250. The fractions and the approximate amount of loaded activity are (a) yeast microsomes, 0.39 pmol/min; (b) NP-40-solubilized microsomes, 0.49 pmol/min; (c) concanavalin A eluate, 2.73 pmol/min; (d) heparin agarose eluate, 2.45 pmol/min; (e) Q-Sepharose eluate, 3.5 pmol/min; (f) hydroxyapatite eluate, 8.0 pmol/min; and (g) hydroxyapatite eluate digested with 0.5 unit PNGaseF, 8.0 pmol/min. In the deglycosylated sample, the oligosaccharyl transferase complex is composed of subunits with apparent molecular weights of 55, 43, 34, and 32 kDa; PNGaseF comigrates with the 34 kDa subunit.

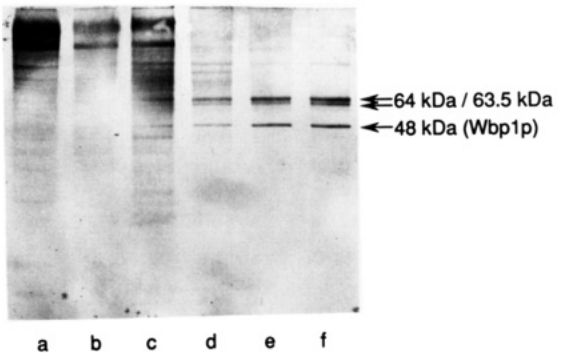


FIGURE 2: Concanavalin A binding of purification fractions. Purification fractions were separated by SDS−PAGE and then transferred to nitrocellulose. Membranes were incubated with concanavalin A−biotin and then developed with avidin-biotinylated alkaline phosphatase. Samples are (a) yeast microsomes, (b) NP-40-solubilized microsomes, (c) concanavalin A eluate, (d) heparin agarose eluate, (e) Q-Sepharose eluate, and (f) hydroxyapatite eluate. The pure fraction contains two glycosylated proteins. The 55 kDa protein exists as two or three glycoforms near 64 kDa, and the 43 kDa Wbp1p protein exists as a 48 kDa glycoprotein.

with sodium phosphate, and activity remained bound to the media in the presence of high NaCl concentrations. However, after the previous three steps of purification, chromatography on hydroxyapatite yielded a modest 2-fold purification and little change in the composition of the protein fraction (see Figure 1).

Oligosaccharyl transferase activity copurified with a tetrameric complex of polypeptides (see Figure 1). The largest polypeptides were near 64 kDa. Up to three bands could be seen at this size; two of them were intensely labeled by concanavalin A blotting, and a third was faintly labeled (see Figure 2); Treatment with PNGaseF yielded one polypeptide band of 55 kDa (see Figure 1). The amino-terminal residue sequences of the glycosylated 64 kDa bands and deglycosylated 55 kDa band were identical (data not shown). The next polypeptide at 48 kDa was identified as Wbp1p by amino-terminal sequencing (see Figure 3A) and immunoblotting with an anti-Wbp1p antiserum (te Heesen et al., 1991) (see Figure 4); this band was also stained intensely

A.	MRTDWNFFFCILLQAIFVVGTQTSRTLVLYDQSTEPLEEY	**Wbp1p**
	TQTSRTLVLYDQxTEPLExY	**48 kDa**
B.	MQFFKTLAALVSCISFVLAYVAQDVH	**Swp1p**
	YVAQDVH	**32 kDa**

FIGURE 3: A. The 48 kDa protein is Wbp1p. The amino-terminal protein amino acid sequence from the 48 kDa polypeptide is identical to the sequence derived from the *WBP1* gene (te Heesen et al., 1991). The first 20 amino acids from the genomic sequence of Wbp1p are likely cleaved by the endoplasmic reticulum signal peptidase. B. The 32 kDa protein is Swp1p. The amino-terminal protein amino acid sequence from the 32 kDa polypeptide is identical to the sequence derived from the *SWP1* gene (te Heesen et al., 1991). The first 19 amino acids from the genomic sequence of Swp1p are also likely cleaved by the signal peptidase.

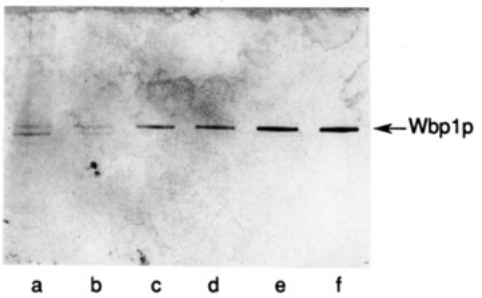


FIGURE 4: Wbp1p is enriched in the purification of the oligosaccharyl transferase. Protein fractions from the purification were separated by SDS−PAGE and transferred to nitrocellulose. The membrane was probed with antiserum raised against Wbp1p (te Heesen et al., 1993) and then developed with an anti-rabbit alkaline phosphatase conjugate. The fractions are (a) yeast microsomes, (b) NP-40-solubilized microsomes, (c) concanavalin A eluate, (d) heparin agarose eluate, (e) Q-Sepharose eluate, and (f) hydroxyapatite eluate. The second band in the first two lanes is a nonspecific, cross-reacting protein stained by the antiserum (te Heesen et al., 1992).

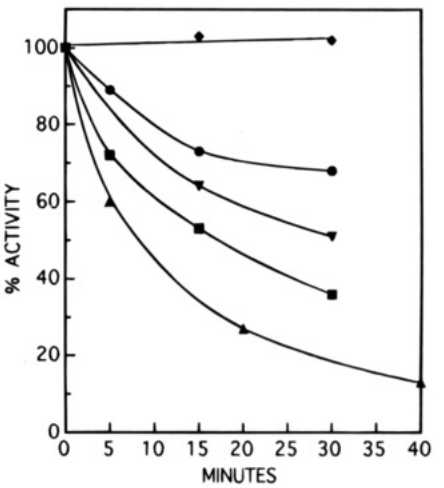


FIGURE 5: Inactivation of oligosaccharyl transferase with methyl methanethiolsulfonate. Enzyme activity was monitored at various concentrations of MMTS (◆, control; ●, 0.5 mM; ▼, 1.0 mM; ■, 2.0 mM; ▲, 4.0 mM).

by concanavalin A and shifted to a molecular weight of 43 kDa upon treatment with PNGaseF (see Figure 1). The first 20 residues of the genomic amino acid sequence from the *WBP1* gene (te Heesen et al., 1991) were not detected by amino-terminal sequencing and were probably cleaved by the endoplasmic reticulum signal peptidase (see Figure 4). The sequence of the amino terminus of the band at 32 kDa identified the protein as Swp1p (see Figure 3B); this protein was not glycosylated (see Figure 2), and amino-terminal sequencing indicated that a 19-residue signal peptide was cleaved by the signal peptidase. A nonglycosylated band at 34 kDa consistently appeared in the purification of the oligosaccharyl transferase (see Figure 1). However, its staining intensity varied in different purifications, and it often stained with significantly less intensity than the other three polypeptides in the complex; this protein has yet to be identified by amino-terminal residue sequencing.

Inactivation by Alkyl Methanethiolsulfonates. To begin the study of the catalytic mechanism of the purified oligosaccharyl transferase, the effects of a series of chemical modifying reagents on the enzyme were examined. NEM, IAA, and IAM did not effectively inactivate the enzyme. However, incubation of Nonidet P-40-solubilized yeast microsomal membrane proteins with MMTS (Smith et al., 1975) at concentrations as low as 100 μM resulted in a substantial loss of oligosaccharyl transferase activity. Purified oligosaccharyl transferase required significantly higher concentrations of 2.5−4 mM MMTS for effective inactivation; inactivation was hindered by the high concentrations of sucrose (140 mM) and PC (0.5 mg/mL) required to maintain the stability of the enzyme.

MMTS selectively modifies the thiol groups of protein cysteine residues and can also modify the amino groups of lysine residues and the imidazole groups of histidine residues under more vigorous conditions (Kluger & Tsui, 1980). Treatment of the oligosaccharyl transferase with MMTS resulted in a time-dependent loss of catalytic activity. The effects of MMTS on the enzyme were concentration-dependent (see Figure 5) and also irreversible as enzyme activity did not recover when the incubation mixture was diluted. Thiol modification with alkyl alkanethiolsulfonates yields a potentially labile disulfide derivative that can often be reversed by the addition of DTT or tributylphosphine (Nishimura et al., 1975). Oligosaccharyl transferase activity was successfully regenerated to approximately 50% of a parallel control by incubation with DTT at 4 °C for several days, indicating that a cysteine residue was modified. Similar experiments with tributylphosphine were unsuccessful as the presence of this reagent resulted in a complete loss in enzymatic activity in a control experiment.

To visualize the inactivation of the enzyme and the derivatization of the polypeptide subunits of the pure complex, the reagent BMTS was synthesized. BMTS is a biotin-labeled molecule with the alkyl methanethiolsulfonate functionality of MMTS; the molecule should both covalently modify the same groups modified by MMTS and provide a label to identify those modified groups. The labeled alkyl alkanethiolsulfonate BMTS showed similar inactivation of the transferase (see Figure 6). Significantly, the rate of inactivation by BMTS decreased when the enzyme was treated in the presence of the substrate 4.4 μM Dol-PP-$(GlcNAc)_2$ (see Figure 7); this substrate concentration was

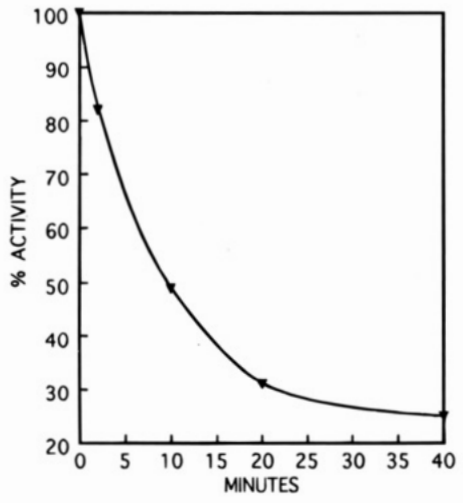


FIGURE 6: Inactivation of oligosaccharyl transferase with 2.5 mM BMTS. Activity was monitored at various times after the addition of 2.5 mM BMTS.

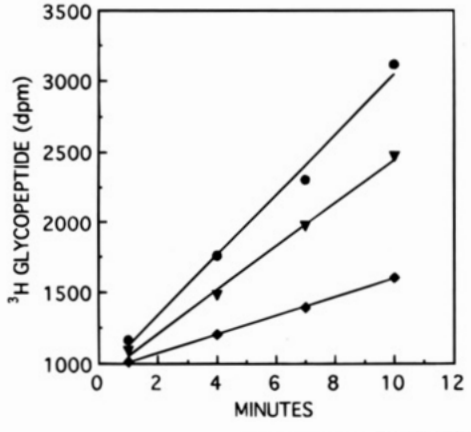


FIGURE 7: Inactivation of oligosaccharyl transferase with 2.5 mM BMTS in the presence and absence of competing Dol-PP(GlcNAc)$_2$. Oligosaccharyl transferase activity was measured 15 min after treatment with methanol or 2.5 mM BMTS (●, methanol, no substrate in preincubation; ▼, 2.5 mM BMTS, 4.4 μM Dol-PP-(GlcNAc)$_2$ in preincubation; ◆, 2.5 mM BMTS, no substrate in preincubation).

comparable to the experimentally determined K_m of the lipid-linked disaccharide for the Nonidet P-40-solubilized yeast enzyme (Sharma et al., 1981). The presence of chitobiose, dolichol phosphate, or excess peptide substrate did not protect the enzyme from inactivation by BMTS. The Dol-PP-(GlcNAc)$_2$ protection of the enzyme from inactivation indicated that alkyl alkanethiolsulfonate modified the transferase at a site that influences the binding of the lipid-linked oligosaccharide substrate.

To identify the modified subunit of the multimeric complex, purified protein was incubated with BMTS and then separated by electrophoresis and transferred to nitrocellulose; the immobilized polypeptides were probed with avidin and biotinylated alkaline phosphatase to visualize biotinylated protein adducts (see Figure 8, top). The only protein modified even after extended treatment with BMTS (2.5 mM, 30 min) was Wbp1p. The increasing intensity of the labeled bands in lanes a−d paralleled the time dependence of the kinetic inactivation of the enzyme. In addition, the derivatization was reversed on exhaustive treatment with DTT under denaturing conditions (lane e) indicating that a cysteine thiol group was modified. When the same samples were

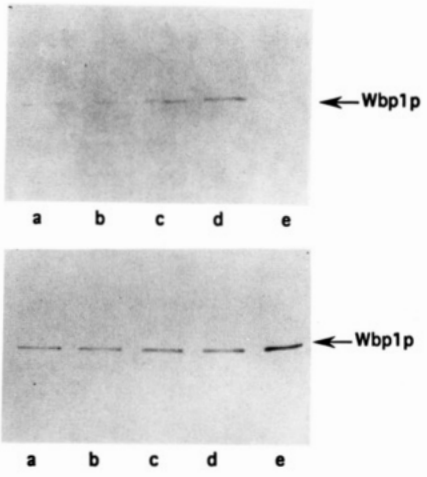


FIGURE 8: Top. Time-dependent labeling of oligosaccharyl transferase with 2.5 mM BMTS. Aliquots of the BMTS-treated enzyme were quenched with cysteine and then separated by SDS-PAGE, transferred to nitrocellulose, and probed with avidin and biotinylated alkaline phosphatase. Each lane contains an aliquot quenched after a different time interval: (a) 0.5, (b), 2.0, (c) 5.0, (d) 30.0, and (e) 30.0 min (treated with DTT). Bottom. Anti-Wbp1p staining of BMTS-treated aliquots. Identical amounts of the same samples were probed with antiserum raised against Wbp1p (te Heesen et al., 1991) and developed with an anti-rabbit IgG alkaline phosphatase conjugate. The samples are (a) 0.5, (b) 2.0, (c) 5.0, (d) 30.0, and (e) 30.0 min (treated with DTT).

treated with antiserum raised against Wbp1p (see Figure 8, bottom), the protein uniquely modified by BMTS was conclusively identified as Wbp1p; in addition, antiserum staining verified that each sample contained the same amount of protein (lanes a−d). The sample treated with DTT stained slightly more intensely (lane e) than the other samples possibly because the reducing sample buffer solubilized the protein more effectively than the nonreducing sample buffer. The labeling of the Wbp1p subunit suggests that the polypeptide is involved in binding of the lipid-linked oligosaccharide substrate and catalysis. In addition, the reversal of the labeling by treatment with DTT indicates that a cysteine residue was modified. Hence, the covalent labeling of at least one of the cysteine residues of Wbp1p abolishes transferase catalysis, and that residue is likely necessary for the binding of the lipid-linked oligosaccharide substrate.

DISCUSSION

The yeast oligosaccharyl transferase has been purified as a tetrameric complex from an extract of microsomal membranes. The mammalian enzyme was also purified as a similar multimeric complex from canine pancreas cells (Kelleher et al., 1992). The largest polypeptide in the yeast complex has a deglycosylated molecular weight of 55 kDa and is glycosylated in at least two positions; the amino-terminal amino acid sequence (Knauer & Lehle, 1994) of this polypeptide shows similarity to ribophorin I, the largest polypeptide of the mammalian complex. The next component of the complex, the Wbp1p protein, has a deglycosylated molecular weight of 43 kDa and is also glycosylated *in vivo*. When the *WBP1* gene product was expressed under the control of the *GAL* promoter, depletion of the gene product correlated with a reduction in oligosaccharyl transferase activity (te Heesen et al., 1992). Wbp1p shows significant amino acid similarity and identity to the mammalian OST48

protein, a component of the mammalian transferase complex (Silberstein et al., 1992). The Swp1p protein is the third component of the complex, and it has a molecular weight of 32 kDa. The depletion of Swp1p from yeast cells also yielded a corresponding reduction in oligosaccharyl transferase activity (te Heesen et al., 1993). Swp1p shows significant amino acid similarity and identity to the carboxy-terminal half of the mammalian ribophorin II, the third component of the trimeric mammalian complex (Kelleher et al., 1994). The final component of the yeast complex has a molecular weight of 34 kDa but has eluded further characterization; it has been hypothesized that this protein is similar to the amino-terminal half of the mammalian ribophorin II (Knauer & Lehle, 1994). Overexpression of the cloned *WBP1* and *SWP1* genes did not increase oligosaccharyl transferase activity in yeast cells (te Heesen et al., 1993); the cloning of the genes encoding the 55 and 34 kDa proteins should enable the complete expression of the enzyme.

The Wbp1p protein is crucial for the catalytic action of the oligosaccharyl transferase. The reagents MMTS and BMTS modified the enzyme at a site that can be protected by the binding of the dolichylpyrophosphoryl oligosaccharide substrate and inactivated the enzyme in a time-dependent and irreversible manner. Visualization of the enzyme after inactivation with the labeled BMTS reagent identified Wbp1p as the uniquely modified polypeptide subunit of the tetrameric complex. The time-dependent kinetic activation of the enzyme was paralleled by a time-dependent increase in the staining intensity of the labeled protein. The combined inactivation and labeling experiment with BMTS showed that Wbp1p contains a residue which is crucial for enzymatic activity and protected by substrate incubation. Wbp1p is thus involved in transferase catalysis and, more specifically, in the binding of the lipid-linked oligosaccharide substrate. Treatment of the BMTS-modified Wbp1p with a disulfide-reducing reagent abolished labeling of the protein and thus identified the labeled group as a cysteine thiol. The sequence of the *WBP1* gene predicts that Wbp1p has three cysteine residues (te Heesen et al., 1991). Site-directed mutagenesis coupled with chemical modification of the protein by BMTS will identify the protected cysteine and provide further insight into the substrate binding of Wbp1p.

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