Structure of the N-Linked Oligosaccharides That Show the Complete Loss of α,1,6-Polymannose Outer Chain from ochl, ochl mnn1, and ochl mnn1 alg3 Mutants of Saccharomyces cerevisiae*

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The periplasmic invertase was purified from Saccharomyces cerevisiae ochl::LEU2 disruptant cells (Δochl), which have a defect in elongation of the outer chain attached to the N-linked core oligosaccharides (Nakayama, K., Nagasu, T., Shimma, Y., Kurofutasu, J., and Jigami, Y. (1992) EMBO J. 11, 2511-2519). Structural analysis of the pyridylaminated (PA) neutral oligosaccharides released by hydrazinolysis and N-acetylation confirmed that the ochl mutation causes a complete loss of the α,1,6-polymannose outer chain, although the PA oligosaccharides (ManαGlcNAc2-PA and ManαGlcNAc2PA), in which one or two α,1,3-linked mannose(s) attached to the endoplasmic reticulum (ER)-form core oligosaccharide (ManαGlcNAc2) were also detected. Analysis of the Δochl mnn1 strain oligosaccharides released from total cell mannoprotein revealed that the Δochl mnn1 mutant eliminates the α,1,3-mannose attached to the core and accumulates predominantly a single ER-form oligosaccharide species (ManαGlcNAc2), suggesting a potential use of this strain as a host cell to produce glycoproteins containing mammalian high mannose type oligosaccharides. The Δochl mnn1 alg3 mutants accumulated ManαGlcNAc2 and ManαGlcNAc2 in total cell mannoprotein, confirming the lack of outer chain addition to the incomplete corelike oligosaccharide and the leaky phenotype of the alg3 mutation. All the results suggest that the OCHl gene encodes an α,1,6-mannosyltransferase that is functional in the initiation of the α,1,6-polymannose outer chain addition to the N-linked core oligosaccharide (ManαGlcNAc2 and ManαGlcNAc2) in yeast.

We have described new temperature-sensitive (ts) Saccharomyces cerevisiae mutants, which exhibit a deficiency in mannose outer chain addition of asparagine (N)-linked oligosaccharides (1). The strains, designated ochl and och2, were obtained by the [3H]mannose suicide technique, and the ochl mutant showed a 2:2 cosegregation with regard to the glycosylation defect and ts growth phenotype. The size of external invertase of the ochl mutant was slightly larger than that of the sec18 mutant, which accumulates the ER-form core oligosaccharide (ManαGlcNAc2) at the non-permissive temperature (2), but it was slightly smaller than that of mnn9 mutant, which contains two mannoses of outer chain attached to the core oligosaccharide (3). Cloning of the OCHl gene, by complementation of ts growth phenotype, and subsequent characterization of the gene product demonstrated that the OCHl gene encodes a novel membrane bound mannosyltransferase (4). The OCHl protein-overproducing cells exhibited about 5-fold greater mannose transfer activity with the mannoprotein prepared from the ochl::LEU2 disruptant cells (Δochl) as an acceptor for the in vitro mannose transfer reaction, while the smaller oligosaccharides, such as α-methyl-mannoside, α,1,6-mannobiose, and mannnotetaose, used to measure conventional α,1,2- or α,1,6-mannosyl transfer, were inactive. Therefore, further studies on the structure of acceptor molecule for the OCHl protein-dependent mannose transfer are required to elucidate the role of OCHl protein in outer chain biosynthesis in yeast. Here we report the structures of N-linked oligosaccharides of external invertase prepared from Δochl cells and those of cell wall mannoprotein prepared from Δochl mnn1 double mutant and from Δochl mnn1 alg3 triple mutant. The structural relationship indicated that the strains containing the Δochl mutation completely lack the α,1,6-polymannose outer chain attached to the core oligosaccharide (ManαGlcNAc2), not only in a specific glycoprotein, invertase, but also more generally in cell wall mannoprotein. The accumulation of predominantly a single oligosaccharide, ManαGlcNAc2, due to the disappearance of α,1,3-linked mannose addition, was also confirmed in the Δochl mnn1 mutant. Together with previous results on the properties of the OCHl protein (4), the results reported herein indicate that the OCHl gene encodes a membrane-bound α,1,6-mannosyltransferase that is functional in the initiation of the polymannose outer chain addition to the N-linked core oligosaccharide.

EXPERIMENTAL PROCEDURES

Strains and Media

S. cerevisiae strain EHA-1C (MATa leu2-3 leu2-112 pep4-3) has been described in a previous paper (1). S. cerevisiae YSS2-1,1B, in which the OCHl gene is disrupted by LEU2 has been described previously (4). S. cerevisiae YSS7-5A (MATa ochl::LEU2 leu2 ura3 his1 his3), YSS7-5C (MATa ochl::LEU2 leu2 ura3 his1 his3), and YSS7-52 (MATa ochl::LEU2 leu2 ura3 his1 his3) were constructed by the standard genetic methods (5) as described (4). S. cerevisiae LB1-10B (MATa mnn1) (6) was obtained from the Yeast Genetic Stock Center, University of California, Berkeley. S. cerevisiae LB347-1C (MATa mnn9) (7), was provided by Dr. W. Tanner (University of Regensburg, Germany). S. cerevisiae PRY90 (MATa alg3-1 ade2-101 ura3-52) (8) was provided by Dr. W. Robbins, Massachusetts Institute of Technology. Cells were grown in YPD medium (2% Bacto-peptone, 1% yeast extract, 2% glucose), supplemented with 0.3 M sorbitol as an osmotic stabilizer.

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Purification of Invertase

YS52-1-1B (Δochl) was grown in a 5-liter jar fermenter containing 3 liters of medium (YPD + 0.3% sorbitol) at 25 °C. When glucose was exhausted by the cells, 0.5% sucrose was added to re-repress the invertase gene expression. The cells were incubated for an additional 4 h and then harvested by centrifugation. Invertase activity was assayed at 30 °C by measuring the release of glucose from sucrose with Glucose CL Test Wako (Wako Chemical Co., Osaka, Japan) in sodium acetate buffer (pH 5.0) according to the supplier's manual.

External (periplasmic) invertase was purified by Neumann and Lam- pen (9) with some modifications. All operations were done at 4 °C. The cells obtained from 12-liter cultures were suspended in 10 mM potassium phosphate buffer (pH 6.5) (buffer A) containing 1.0 mM phenylmeth- ylsulfonyl fluoride and broken in a French pressure cell press (SLM, Amino) at 12,000 p.s.i., and the homogenates were centrifuged to remove the cell debris. Streptomycin sulfate (1% final) was added to remove nucleic acids. Ammonium sulfate was then added to the supernatant until 75% saturation to precipitate non-glycosylated internal invertase and most of the contaminating proteins. After centrifugation, the supernatant was dialyzed against the buffer A. Ten grams of DEAE-Sephadex A-50 resin, pre-equilibrated with buffer A, was added to the dialysate. After the invertase activity remaining in solution was reduced to less than 10% of the initial, the liquid was removed, and the resin was packed in a column (2.5 × 35 cm) and washed with two volumes of the same buffer containing 0.1 M NaCl. External glyco- sylated invertase was then eluted with the buffer containing 0.2 M NaCl, and the fractions containing enzyme activity were pooled and dialyzed against deionized water. The dialysate was lyophilized, dissolved in deionized water, and applied to a Sephadex G-200SF column (2.0 × 95 cm) equilibrated with buffer A containing 0.2 M NaCl. The column was eluted with the same buffer, and the invertase-containing fractions were pooled and lyophilized.

The sample thus obtained gave a discrete protein band on SDS-PAGE (10) using a 4–20% gradient gel (SDS-PAGE Plate 4/20, 10 cm × 10 cm, Daichi Pure Chemicals, Tokyo, Japan) with Coomassie Brilliant Blue staining. Table 1 summarizes the purification steps from Δochl cells (YS52-1-1B strain) obtained from a 12-liter culture. The yield of purified glycosylated invertase was 2 mg from 180 g of wet cells. The above process was repeated four times to obtain sufficient invertase to analyze its oligosaccharide structure.

Hydrazinolysis and N-Acetylation

About 8 mg of purified external invertase, isolated from Δochl cells according to the above procedure, was subjected to hydrazinolysis to release external mannoprotein (14). The cells were incubated in a gas-phase hydrazinolysis apparatus (Hydrasclub S-204, Honen Oil Co., Tokyo, Japan). The hydrazinolysis and N-acetylation were performed at 60 °C for 18 h at 37 °C, and the reaction was stopped by boiling for 5 min and centrifuged to remove insoluble material. Hydrolyzation and N-Acetylation

The oligosaccharides released from proteins, either by hydrazinolysis or by glycopeptidase A, were labeled with 2-aminopyridine to facilitate the sensitive detection by fluorescence (18, 19). Hydrazinolysis was carried out by using the commercially available reagent kit (Takara Shuzo Co., Kyoto, Japan). The pyridylaminated derivatives (PA oligo- saccharides) were loaded on a Toyopearl HW-40F column (1.0 × 40 cm) ( Tosoh Co., Tokyo, Japan), which was pre-washed with 10 mM sodium acetate buffer (pH 7.0), and eluted with the same buffer containing 1 mg of mannoprotein in 10 μl of the same buffer. The mixture was incubated for 18 h at 37 °C, and the reaction was stopped by boiling for 5 min and centrifuged to remove insoluble material.

Table 1 Purification of the external invertase synthesized by Δochl cells at 25 °C

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units x 10^{-3})</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cruude cell extract</td>
<td>5.00</td>
<td>765.5</td>
<td>153</td>
<td>100</td>
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<tr>
<td>2. Streptomycin sulfate</td>
<td>4.20</td>
<td>665.6</td>
<td>157</td>
<td>87</td>
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<tr>
<td>3. Ammonium sulfate</td>
<td>500</td>
<td>757.6</td>
<td>384</td>
<td>75</td>
</tr>
<tr>
<td>4. DEAE-Sephadex</td>
<td>9</td>
<td>333.3</td>
<td>3700</td>
<td>44</td>
</tr>
<tr>
<td>5. Sephadex G-200SF</td>
<td>4.5</td>
<td>20.6</td>
<td>4560</td>
<td>57</td>
</tr>
</tbody>
</table>

* Protein was determined according to Bradford (11) using bovine serum albumin as a standard for steps 1–4. Protein concentration for step 5 was determined from the absorbance at 280 nm (ε_{280} = 22.5) (12).

The separation of PA oligosaccharides was carried out by HPLC using a Shimadzu LC-6A chromatograph system equipped with a fluorescence spectrometer RF-530.

Anion Exchange HPLC—Since some of N-linked oligosaccharides on invertase were phosphorylated in S. cerevisiae (20), anion exchange Cos- mogen QA column (0.5 × 7.5 cm, Nakarai Tesque, Kyoto, Japan) was used to separate neutral oligosaccharides from the phosphorylated ones. The column was previously equilibrated with 10 mM sodium acetate buffer (pH 8.0) at a flow rate of 0.5 ml/min. At 20 min after a sample injection, the buffer concentration was increased linearly up to 45 ml/min for 15 min. The neutral oligosaccharide fractions were eluted within 17 min after the sample injection and pooled for further analysis. PA oligosaccharides were detected by fluorescence (Ex = 310 nm and Em = 380 nm).

Size-fractionation HPLC—The retention time of oligosaccharide largely depends on the number of sugar residues in the amine-modified column chromatography (21). Size-fractionation HPLC was performed with Aasahi Pak NH2P-50 (0.46 × 25 cm) (Asahi Chemical Co., Tokyo, Japan) at a flow rate of 1.0 ml/min. Two solvents, A and B, were used. Solvent A was composed of 200 mM acetic acid adjusted with triethylamine (pH 7.3) and acetonitrile (35:65, v/v). Solvent B was composed of 200 mM acetic acid adjusted with triethylamine (pH 7.3) and acetonitrile (50:50, v/v). The column was equilibrated with solvent A. After the sample injection, the proportion of solvent B was increased linearly up to 100% for 50 min. PA oligosaccharides were detected by fluorescence (Ex = 310 nm and Em = 380 nm).

Reverse-phase HPLC—The reverse-phase HPLC was performed with C18Spherisorb 5 μm (0.46 × 15 cm) (Nakarai Tesque) at a flow rate of 1.0 ml/min. Two solvents, C and D, were used. Solvent C was 10 mM sodium phosphate buffer (pH 3.8), and solvent D was 10 mM sodium phosphate buffer (pH 3.8) containing 3.1% 1-butanol. The column was equilibrated with a mixture of solvent C and D (95:5; v/v). At 50 min after the sample injection, the ratio of solvent D was increased linearly up to 100% for 90 min. Samples were detected by fluorescence (Ex = 320 nm and Em = 400 nm).
Partial acetylation of PA oligosaccharide (23) was carried out to release α-1,6-linked mannose specifically. A 5-nmol sample of PA oligosaccharide was peracetylated with a mixture (40 μl) of acetic anhydride and pyridine (1:1) at 100 °C for 15 min in a sealed glass tube. After removing excess reagent by evaporation, 20 μl of a mixture containing acetic anhydride-acetic acid-sulfuric acid (10:10:1) was added. The tube was heated at 70 °C for 15 min. Four microliters of pyridine was added, and the solution was dried by evaporation. Then 0.4 ml of saturated sodium bicarbonate solution was added to the residue, and the products were extracted five times with 0.5 ml each of chloroform. After evaporation of the combined chloroform extract, the sample was subjected to hydrazinolysis and N-acetylation as described, and the PA oligosaccharide sample was analyzed by HPLC.

**FAB-MS**

Fast atom bombardment mass spectroscopy (FAB-MS) was carried out using a JMS-HX110 mass spectrometer (JEOL Co., Tokyo, Japan) equipped with an FAB ion source operated with xenon at 10 kV of accelerating voltage. The matrix used was a mixture containing acetic alcohol and glycerol (6:4). Samples (1–2 μg of PA oligosaccharide) were desalted by HPLC using either an Aeshpak N12P-50 (0.46 × 25 cm) or a Tosoh TSKgel Amide-80 column (0.46 × 20 cm) at a flow rate of 0.5 ml/min with a mixture of acetonitrile and water (1:1).

**High Resolution 1H NMR**

1H NMR spectra of PA oligosaccharide were measured on a JNM-GX400 (JEOL Co.) at 50 °C. Samples (50–300 μg, except for 18 μg of authentic Man9GlcNAc2-PA) were dissolved in 99.96% D2O and lyophilized. After three repetitions of the above procedure, the samples were finally dissolved in 500 μl of 99.996% D2O and lyophilized. The chemical shifts (δ) are expressed in parts/million (ppm) downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but they were actually measured by reference to internal acetone (δ 2.247 ppm). Authentic PA oligosaccharides were purchased from Takara Shuzo Co.

**Double and Triple Mutant Preparation**

Double and triple mutants were constructed by sporulating the appropriate heterozygous diploids and isolating the haploid mutant clones after tetrad dissection (5). In crosses between YN1-28A and YS57-5A, and between YN1-28B and PRY90 (alg3), the sporulation ratio was about 10%, and the segregant carrying the mutation was not obtained. Thus, mutants were selected by the Western blot analysis using anti-α-1,3-mannan antibody (kindly supplied by Dr. R. Schekman, University of California, Berkeley) and these strains were designated YN1-28A, YN1-28B, YN2-12A, YN2-14A and YN2-15C, were isolated from the cross between YN4-18A, carrying och1 mnn1, and och1 mnn1 alg3 triple mutant (24), each ladder band in och1 mnn1 cells reflects the number of sugar chains attached to invertase protein, which has 13 potential N-glycosylation sites in wild type S. cerevisiae (25). The invertase from alg3 cells gave a broad smear (Fig. 1, lane 4), the migration range of which is similar to that of wild type cells, indicating an addition of polymannose outer chain. The mobility of invertase from och1 mnn1 alg3 double mutant cells is slightly faster than that from och1 cells (Fig. 1, lanes 2 and 5), suggesting a shorter oligosaccharide length in och1 alg3 cells than in och1 cells. Furthermore, the och1 mnn1 alg3 triple mutant produced a still smaller invertase molecule (Fig. 1, lanes 3, 5, and 6) as a result of accumulation of three independent mutations that affect N-linked oligosaccharide biosynthesis.

**N-Linked Neutral Oligosaccharide Length**—The size of N-linked neutral oligosaccharides released by hydrazinolysis from the external invertase of och1 cells was analyzed by size-fractionation HPLC. The PA oligosaccharides were separated into three major peaks (peaks 1–3) with some other minor peaks (Fig. 2A). The elution time of peak 1 (21.8 min) is identical to that of authentic Man9GlcNAc2-PA (PA Sugar Chain 019, Takara). Judging from the elution time of PA glucose oligomers (Honen Oil Co.), peaks 2 (25.1 min) and 3 (28.3 min) were estimated to correspond to Man8GlcNAc2-PA and Man10GlcNAc2-PA, respectively. The och1 cells cultivated at the permissive temperature (25 °C) completely lacked the larger oligosaccharides that were observed in invertase from baker's yeast and from the och1 ts mutant under the permissive temperature (1).

The PA oligosaccharides released by glycopeptidase A from the cell wall mannanoprotein of och1 cells showed a similar elution pattern on size-fractionation HPLC, except for some minor peaks at 50–60 min (data not shown). Since the minor peaks were not observed in the PA oligosaccharide from external invertase of och1 cells, but were seen in the total mannanoprotein from the mnn9 mutant, these minor products may be derived from mannanprotein during the high temperature extraction of cell wall material.

The oligosaccharide from och1 mnn1 double mutant mannanoprotein contained predominantly a single species, corresponding to Man9GlcNAc2-PA (Fig. 2B, peak 4), suggesting the loss of α-1,3-mannose addition due to the mnn1 mutation. A minor component (10% of Man9GlcNAc2-PA) was observed at the elution time of 24.2–25.5 min, which corresponds to that of Man8GlcNAc2-PA (see "Discussion").

The elution pattern of PA oligosaccharides from och1 mnn1 alg3 triple mutant mannanprotein (Fig. 2C) reveals two major peaks (peaks 5 and 6). Judging from the elution time of PA glucose oligomers, peak 5 (12.3 min) corresponds to...
Oligosaccharide Structure of Invertase from Aochl Cells

Each PA oligosaccharide fraction corresponding to peaks 1–3 in Fig. 2A from Aochl cells was collected individually and analyzed on reverse-phase HPLC to examine its purity. Each showed a single peak on reverse-phase HPLC, suggesting a single component (data not shown). The elution time of component 1 (31.6 min) was in accordance with that of the authentic ER-form Man₇GlcNAc₂-PA sample (PA Sugar Chain 019, Takara). Components 1–3 were analyzed by positive ion FAB-MS to give positive molecular ion peaks at m/z 1,800, indicating the structure of Man₇GlcNAc₂-PA (calculated 1,812.5) and Man₈GlcNAc₂-PA (calculated 1,798.7), respectively. These data were in accordance with the estimated structure of the component 1 as an ER-form core PA oligosaccharide. Based on the estimated structure of component 1, component 2 was estimated to be a Man₇GlcNAc₂-PA in which one mannose residue, not connected by α-1,2 linkage, is attached to the Man₇GlcNAc₂-PA core oligosaccharide at the mannose of position 11 in Fig. 3A. In addition, component 3 was estimated as Man₉GlcNAc₂-PA in which one additional mannose is attached to the above Man₇GlcNAc₂-PA component at the mannose of position 9 in Fig. 3A.

The NMR spectrum for authentic Man₅GlcNAc₂-PA (ER-form core PA oligosaccharide, PA Sugar Chain 019, Takara) and those for Man₇GlcNAc₂-PA, Man₈GlcNAc₂-PA, and Man₉GlcNAc₂-PA samples from invertase of Aochl cells are shown in Fig. 3 (panels A–D, respectively). Chemical shifts (δ ppm) for C1 and C2 protons are summarized in Table II, and linkage assignments (Fig. 3 and Table II) were based on the reported chemical shift assignments (26, 27). The spectrum for the Man₅GlcNAc₂-PA sample from Aochl cells (Fig. 3B) is identical to that for the authentic ER-form Man₅GlcNAc₂-PA (Fig. 3A). In the spectrum of the Man₇GlcNAc₂-PA sample from Aochl cells, one additional C1-H signal was observed at 5.14 ppm, which is characteristic of the anomeric proton of the unsubstituted α-1,3-linked mannose (Fig. 3C). The signal at 5.14 ppm was doubled in intensity in the spectrum for the Man₉GlcNAc₂-PA sample, indicating the attachment of one additional α-1,3-linked mannose residue to the Man₉GlcNAc₂-PA structure. Since the α-1,2-mannosidase digestion of Man₇GlcNAc₂-PA yielded Man₈GlcNAc₂-PA, this α-1,3 linkage must be the mannose at position 11 in Fig. 3A, confirming the structure of Man₇GlcNAc₂-PA as shown in Fig. 3C. Considering the resistance of the Man₇GlcNAc₂-PA sample to α-1,2-mannosidase digestion, the oligosaccharide can be assigned the structure shown in Fig. 3D. The oligosaccharide structures shown in Fig. 3 (panels A–D) are in good agreement with the chemical shifts assigned in Table II.

Oligosaccharide Structure of Mannoproteins from Aochl mnn1 Double Mutant—The major PA oligosaccharide obtained from Aochl mnn1 cells on size-fractionation HPLC (peak 2 in Fig. 2B) showed a single peak on reverse-phase HPLC, designated as component 4 (data not shown). The elution time of component 4 was identical to that of authentic Man₅GlcNAc₂-PA and the molecular mass was confirmed by FAB-MS, which gave m/z 1,800, as estimated for Man₅GlcNAc₂-PA (calculated 1,798.7). After α-1,2-mannosidase digestion of component 4, the peak was shifted from M₈ to M₅ position on size-fractionation HPLC. The NMR spectrum of this component 4 (Fig. 3E and Table II) was identical to that of authentic Man₅GlcNAc₂-PA (Fig. 3A). From these data, we conclude that the Aochl mnn1 cells predominantly produced the ER-form Man₅GlcNAc₂ as a neutral oligosaccharide.

Oligosaccharide Structure of Mannoproteins from Aochl mnn1 alg3 Triple Mutant—Each PA oligosaccharide fraction, corresponding to peaks 5 and 6 in Fig. 2C, from Aochl mnn1 alg3 mutant cells showed a single peak on reverse-phase HPLC (components 5 and 6, respectively). The elution time on reverse-phase HPLC of component 6 was same as that of the authentic ER form of Man₈GlcNAc₂-PA. The ratio of components 5 and 6 was 1:1.4. It is reported that the alg3 sec18 mutant produced external invertase containing Man₉GlcNAc₂ and Man₈GlcNAc₂ N-linked oligosaccharides after the temperature shift (28). In accordance with the reported oligosaccharide structure of alg3 sec18 (28), we assumed that component 5 produced by Aochl mnn1 alg3 mutant cells must be the...
Man9GlcNAc2-PA shown in Fig. 4A, and component 6 may be the ER-form Man9GlcNAc2-PA shown in Fig. 3A. To confirm these structures, components 5 and 6 were subjected to α-1,2-mannosidase digestion and partial acetylation. After α-1,2-mannosidase digestion, the component 5 peak was shifted from the M5 position (12.0 min) to the M3 position (7.2 min) (Fig. 4, A and B) on size-fractionation HPLC. The molecular masses of component 5 (M5) and enzyme digestion product (M3) were confirmed by FAB-MS, which gave m/z 1,313, as estimated for Man9GlcNAc2-PA (calculated 1,312.5), and m/z 989, estimated for Man9GlcNAc2-PA (calculated 988.4), respectively. After partial acetylation, which specifically removes α-1,6-linked mannose residues, the peak of component 5 (12.0 min) was shifted to the M4 position (9.4 min) (Fig. 4, C and D). This M4 product showed a protonated molecular ion at m/z = 1,152, indicating the formation of Man4GlcNAc2-PA (calculated 1,150.4). These data confirmed the structure of component 5 as the Man9GlcNAc2-PA shown in Fig. 4A. In contrast, component 6 was shifted from the M8 position (21.5 min) to the M5 position (12.0 min) after α-1,2-mannosidase digestion, and from the M8 position (21.5 min) to the M4 position (9.4 min) after partial acetylation (data not shown). The molecular mass of M5 product
The OCH1 protein encodes a novel membrane-bound mannosyl-
transferase that transfers mannose from GDP-[14C]mannose to
the oligosaccharide isolated from mannanprotein from
Δoch1 cells. At that time, we assumed that the OCH1 protein might be
functional not in the initiation of a-1,6-polymanose addition to
MαGlcNAc2, but rather in the elongation of outer chain from
MαGlcNAc2, a conclusion that was based on the accumulation
of a major OA oligosaccharide corresponding to
MαGlcNAc2-PA and the lack of mannosyl transfer to the
"MαGlcNAc" in an in vitro reaction (4). The present results,
however, are not consistent with the previous assumption,
because all of the oligosaccharides identified in Δoch1 cells lack
α-1,6-mannose attached to the MαGlcNAc2 core oligosaccha-
ride. Therefore, we have examined whether the OCH1 protein
can use MαGlcNAc2-PA and MαGlcNAc2-PA as mannosyl procesors. The microsomal membrane fraction prepared from
OCH1 protein-overproducing cells was found to transfer man-
nose efficiently to MαGlcNAc2-PA to form MαGlcNAc2-PA
and to MαGlcNAc2-PA to form MαGlcNAc2-PA, and this
in vitro reaction was diminished in the membrane fraction from
Δoch1 cells. In addition, in the previous study (4), we used
MαGlcNaoH (octamannosyl-N-acetylglucosaminitol) as ac-
ceptor, which was incorrectly called MαGlcNAc (octamanno-
syl-N-acetylglucosamine). Now, we have confirmed that
MαGlcNaoH does not serve as an acceptor for the OCH1
protein-dependent mannosyl transfer reaction, whereas
MαGlcNac does. Accordingly, it now becomes clear that the
OCH1 gene encodes an α-1,6-mannosyltransferase that is func-
tional in the initiation of mannosyl outer chain addition to the
ER-form core oligosaccharide, as shown in Fig. 5. Romero and
Herscovics (29, 30) reported the purification and characteriza-
tion of α-1,6-mannosyltransferase, which is certain to be the
same enzyme encoded by OCH1 gene. Although we cannot ex-
clude the possibility that the OCH1 protein may also function
in the subsequent elongation to form a polymannose outer
chain, this seems unlikely because reaction products larger
than one mannosyl addition were not observed in our in vitro
mannosyl transfer experiments even with a prolonged incuba-
tion period. Addition of α-1,3-mannose is known to occur with the core-
like oligosaccharide to which mannosyl (Man-α-1, 2-Man-α-1,
β-) is added to the ER-form oligosaccharide (compound E in
Fig. 5), in wild type invertase (28), carboxypeptidase Y (91), and

**DISCUSSION**

We have determined the structures of the N-linked neutral
oligosaccharides attached to invertase in Δoch1 cells, as well as
those of the neutral oligosaccharides attached to total manno-
protein from Δoch1 mnn1 and Δoch1 mnn1 alg3 mutants. The
results demonstrate that the OCH1 gene disruption leads to
loss of function to add the α-1,6-polymanose outer chain to the
ER-form core oligosaccharide. Previously (4), we reported that
the OCH1 gene encodes a novel membrane-bound mannosyl-
transferase that transfers mannosyl from GDP-[14C]mannose to
the oligosaccharide isolated from mannanprotein from Δoch1
cells. At that time, we assumed that the OCH1 protein might be

**TABLE II**

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<tr>
<th>Residue</th>
<th>Authentic M8</th>
<th>Δoch1 M8</th>
<th>Δoch1 M9</th>
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*Intensities for individual signals of C2-H could not be calculated due to the overlap of signals.*

![Fig. 4](image-url)  
Fig. 4. Elution profile on size fractionation HPLC of PA oligo-
saccharides released from Δoch1 mnn1 alg3 mannanprotein. A and C, component 5 corresponding to peak 5 in Fig. 2C; B, after α-1,2-mannosidase digestion; D, after partial acetylation.

from M8 sample after α-1,2-mannosidase digestion and that of
M4 product from M8 sample after partial acetylation were also
confirmed by FAB-MS (data not shown), demonstrating the ER
form of MαGlcNAc2-PA (Fig. 3A) as component 6 (Fig. 2C).

![Table II](image-url)

**Fig. 2** Elution profile on size fractionation HPLC of PA oligo-
saccharides released from Δoch1 mnn1 alg3 mannanprotein. A and C, component 5 corresponding to peak 5 in Fig. 2C; B, after α-1,2-mannosidase digestion; D, after partial acetylation.

![Table II](image-url)
ochl, ochl mnnl, and ochl mnnl alg3 Yeast Oligosaccharides

Fig. 5. N-Linked oligosaccharide biosynthesis in S. cerevisiae and deficient steps caused by the ochl and mnnl mutations, together with the proposed steps caused by the mnn9 mutation (31).

It is noteworthy that the α-1,3-mannose addition to the core oligosaccharide occurs even in the absence of this mannobiose unit (see compounds B and C in Fig. 5). The first α-1,3-mannose addition occurs at the terminus of the α-1,3-linked mannotriose branch (position 11 of compound A in Fig. 5), and the subsequent α-1,3-mannose addition occurs at the end of α-1,6-linked mannotetraose branch (position 9 of compound A in Fig. 5), which is consistent with the previous results for the oligosaccharides from both wild type and mnn2 mnn9 mannoproteins (26, 32). This order of α-1,3-mannose addition to the core oligosaccharide may suggest the higher affinity of α-1,3-mannosyltransferase encoded by MNN1 gene (33) to add the mannose at position 11 of compound A in Fig. 5 rather than at position 9 of compound A in Fig. 5.

We have clarified that the ochl mnnl mutant cells produce predominantly the Man8GlcNAcz core oligosaccharide due to the lack of α-1,3-mannosyltransferase encoded by MNN1 gene (33) to add the mannose at position 11 of compound A in Fig. 5. The ochl mnnl alg3 cells accumulated both the Man8GlcNAcz-PA ER-form core oligosaccharide (Fig. 3A) and the Man5GlcNAcz-PA oligosaccharide (Fig. 4A). The alg3 mutant is known to accumulate lipid-linked ManαGlcNAc2 before transfer to the Asn residue of protein (8, 34). It is likely that the Man5GlcNAcz-PA, which lacks both the Man-α-1,2-Man-α-1,6-linked mannobiose and the Man-α-1,3-linked mannose of compound A in Fig. 5, was caused by transfer to proteins of the incomplete lipid linked Man,GlcNAc2. The formation of ManαGlcNAc2 is assumed to be caused by a leaky phenotype of the alg3 mutation, as reported for the oligosaccharide structure from alg3 sed8 invertase (28). The molar ratio of ManαGlcNAc2 to Man8GlcNAcz was 1:1.4 in ochl mnnl alg3 mutant, whereas it was 5:1 in alg3 sec18 mutant. This may reflect the different growth conditions, because ochl mnnl alg3 manno-protein was obtained from cells cultivated at 28 °C, whereas invertase from alg3 sec18 cells was obtained by a 3-h derepression at 37 °C after cultivation at the permissive temperature due to the ts phenotype of the sec18 mutant (28). This suggests that the accumulation of the incomplete precursor of N-linked oligosaccharides may be temperature-dependent in the alg3 mutant, although the alg3 mutant itself is not ts for cell growth.

mnn9 mannoproteins (32) to form compounds F and G in Fig. 5. It is noteworthy that the α-1,3-mannose addition to the core oligosaccharide occurs even in the absence of this mannobiose unit (see compounds B and C in Fig. 5). The first α-1,3-mannose addition occurs at the terminus of the α-1,3-linked mannotriose branch (position 11 of compound A in Fig. 5), and the subsequent α-1,3-mannose addition occurs at the end of α-1,6-linked mannotetraose branch (position 9 of compound A in Fig. 5), which is consistent with the previous results for the oligosaccharides from both wild type and mnn2 mnn9 mannoproteins (26, 32). This order of α-1,3-mannose addition to the core oligosaccharide may suggest the higher affinity of α-1,3-mannosyltransferase encoded by MNN1 gene (33) to add the mannose at position 11 of compound A in Fig. 5 rather than at position 9 of compound A in Fig. 5.

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The formation of mannose outer chain in the alg3 mutant (Fig. 1, lane 4) was diminished in Δoch1 mnn1 alg3 cells (Fig. 1, lane 6), which suggests that the OCH1 protein is functional in the initiation of outer chain elongation of Man5GlcNAcβ as well as Man5GlcNAcβ. The in vitro experiment supports this assumption by demonstrating that extracts from OCHl protein-overproducing cells have an activity to transfer mannose to Man5GlcNAcβ-PA to form Man5GlcNAcβ-PA.2 In conclusion, our results suggest that the OCH1 gene encodes an α-1,6-mannosyltransferase that is functional in the initiation of α-1,6-polymannose outer chain addition to the N-linked core oligosaccharide (Man5GlcNAcβ and Man6GlcNAcβ) in yeast. Further studies on the substrate specificity and kinetic properties of OCH1 protein are in progress.

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REFERENCES