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Revision of the oligosaccharide structures of yeast carboxypeptidase Y

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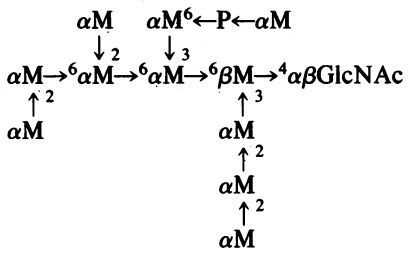
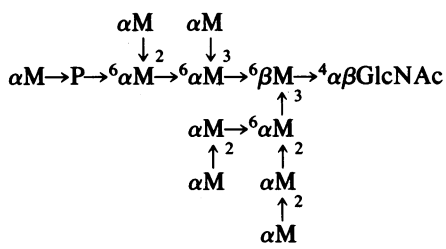
ABSTRACT **The N-linked oligosaccharides from baker's yeast carboxypeptidase Y were analyzed by ^{1}H NMR and specific mannosidase digestion and found to be identical to those from the *Saccharomyces cerevisiae mnn9* mutant bulk mannoprotein. The results support the view that the *mnn* mutants make oligosaccharides that are a true reflection of the normal biosynthetic pathway and confirm that a recently revised yeast oligosaccharide structure is applicable to wild-type mannoproteins.**

Baker's yeast carboxypeptidase Y (CPY) is a well-characterized glycoprotein (1, 2) that has assumed importance as a vacuolar marker enzyme (3) for studies on protein transport and localization (4). Its amino acid sequence, inferred, in part, from the gene sequence (5), reveals four potential glycosylation sites, which agrees with the earlier conclusion that the protein contains four N-linked oligosaccharides (6). In 1981, Ballou and colleagues (7) reported the existence of a phosphorylated N-linked oligosaccharide in baker's yeast CPY and suggested its structure was that of structure I.



Structure I

In more recent investigations on the bulk mannoprotein of *Saccharomyces cerevisiae mnn* mutants, however, we have established both a different structure for the oligosaccharide and a new location for the mannosyl phosphate group as shown by structure II (8).



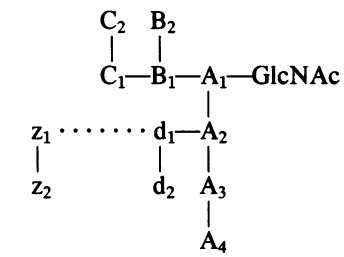
Structure II

The question arises whether structure I is characteristic of a wild-type yeast oligosaccharide and structure II is formed only in the *mnn* mutants. To resolve this uncertainty, we have reinvestigated baker's yeast CPY oligosaccharides and find that they are identical to those we reported for the *mnn9* mutant (8–11). This finding requires a revision of the oligosaccharide structures we reported previously for CPY (7) and suggests that all *S. cerevisiae* mannoproteins have the same general carbohydrate structure with regard to the positions of phosphorylation and the point of attachment of the outer chain.

MATERIALS AND METHODS

CPY, homogeneous by gel electrophoresis, was isolated from baker's yeast (2) and was material that remained from a previous study (7). The sources of all chemicals and materials as well as the procedures for enzyme digestions, for oligosaccharide isolation and dephosphorylation, for chromatography, and for ^{1}H NMR have been reported (8–11). Anion-exchange HPLC was performed on a Dionex BioLC carbohydrate system (9, 12). Bacterial alkaline phosphatase (Sigma type III-N) and human prostatic acid phosphatase (Calbiochem) were used.

Convention for Identifying Mannose Units in Yeast Oligosaccharides. In structure III, the letters stand for mannose units in which the horizontal bonds, with the exception of the linkage to GlcNAc, represent α1→6 linkages, and the vertical bonds represent α1→2 and α1→3 linkages.



Structure III

Mannose units derived from the lipid-linked oligosaccharide precursor are shown in uppercase letters, and those units added during processing are in lowercase letters. The dotted line indicates an extension of the α1→6-linked outer chain; but, when the outer chain is extended, d_2 is not added and an equivalent α1→2-linked mannose (z_2) is present at the end of the outer chain. Mannoses C_1, A_3, and z_1 are sites of phosphorylation (10).

Characteristic differences in the H-1 (anomeric proton) chemical shifts allow us to distinguish between most of the mannoses in this structure (11, 13). The shifts are as follows: A_1, δ4.77; A_2, δ5.32; A_3, δ5.29; A_4, δ5.03; B_1, δ4.87; B_2, δ5.08/5.11; C_1, δ5.125; C_2, δ5.04; d_1, δ5.125; and d_2, δ5.04/5.05. Thus, the terminal α1→2-linked mannoses A_4, C_2, and d_2 (or z_2) all have slightly different chemical shifts. The

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Abbreviation: CPY, carboxypeptidase Y.

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signals for B_2 and d_2 are split by interaction with the GlcNAc anomers (14). When substituted in position 2, C_1 and d_1 have identical shifts but, when unsubstituted, C_1 shifts to δ4.92 and d_1 to δ4.90. When A_4 is removed, A_3 shifts to δ5.04; when A_3 is removed, A_2 shifts to δ5.10. When the outer chain is elongated, the signals for z_1 and z_2 replace and are identical with those for d_1 and d_2.

RESULTS

Isolation of Oligosaccharides. About 220 mg of CPY (33 mg of carbohydrate) was incubated with endoglucosaminidase H (21 mU in four portions over a 5-day period). The digest was boiled to coagulate the protein, and the solution was fractionated on a Bio-Gel P-4 column (2 × 190 cm) by elution with 0.1 M ammonium acetate/0.2% sodium azide (10). A small amount of protein and carbohydrate was eluted near the void volume of the column, followed by a peak of phosphorylated oligosaccharides (17 mg of carbohydrate, 70% of total) and a smaller peak of neutral oligosaccharides (7.8 mg of carbohydrate, 30% of total).

The phosphorylated oligosaccharides were fractionated on a QAE-Sephadex column by gradient elution with NaCl/2 mM Tris base (10, 15) to yield a monophosphate derivative at 25 mM salt and a diphosphate derivative at 70 mM salt. Each derivative was desalted on a column of Bio-Gel P-4 and lyophilized to give 7.5 mg of monophosphate diester and 4.5 mg of diphosphate diester, based on the carbohydrate phenol-sulfuric acid assay.

The oligosaccharide compositions of the neutral fraction and the dephosphorylated monophosphate fraction were compared by anion-exchange HPLC, which gave the patterns in Fig. 1. The neutral fraction (Fig. 1*A*) showed four major and two minor peaks that were assigned molecular sizes of $Man_9GlcNAc$ to $Man_{14}GlcNAc$ inclusive, based on a comparison with oligosaccharides from the *mnn9* mutant (11, 12). The identity of the retention times of peaks B–F with the *mnn9* mutant oligosaccharides of 10–14 mannoses (Fig. 1*B*) suggested that the corresponding homologs in the two preparations could be identical.

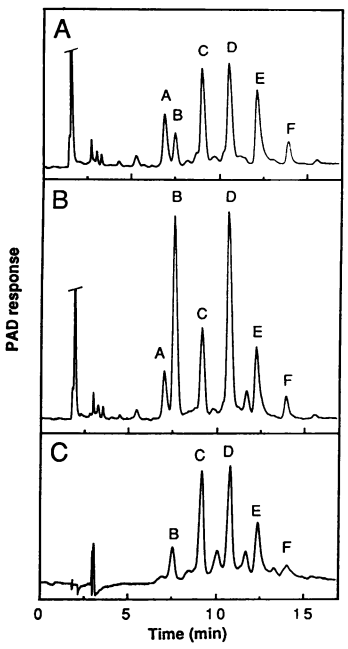
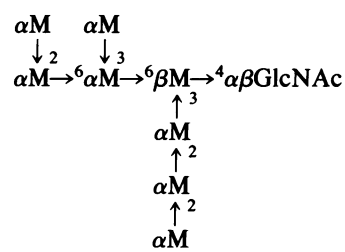


FIG. 1. Oligosaccharide comparison by anion-exchange HPLC. (*A*) CPY neutral oligosaccharides in which peaks A–F are $Man_{9-14}GlcNAc$, respectively. (*B*) CPY neutral oligosaccharide mixture to which $Man_{10}GlcNAc$ from the *mnn1 mnn2 mnn9* mutant (8) and $Man_{12}GlcNAc$ from the *mnn2 mnn9* mutant (11) were added. (*C*) Dephosphorylated monophosphate oligosaccharide from CPY. Peaks B and D in *B* correspond exactly to the two added *mnn* mutant oligosaccharides. In an experiment not shown, peaks C and E were enhanced by addition of the *mnn2 mnn9* $Man_{11}GlcNAc$ and $Man_{13}GlcNAc$. Peak A is absent from *C*, suggesting that this $Man_9GlcNAc$ is not phosphorylated. Minor uncharacterized oligosaccharide peaks are seen in *B* and *C*. The eluent was 100 mM NaOH with a 25-min gradient from 50–130 mM sodium acetate. PAD, pulsed ampometric detector.

Fractionation and Characterization of the Neutral Oligosaccharides. The neutral oligosaccharide fraction was rechromatographed on a Bio-Gel P-4 column (2 × 190 cm) by elution with water, which yielded four peaks and two shoulder fractions (Fig. 2), each of which corresponded to one of the HPLC peaks of the mixture. The fractions were collected separately to give 0.5–1.1 mg of each. The corresponding oligosaccharides are given identical letters in Figs. 1 and 2.

The ^1H NMR spectra (Fig. 3) of the neutral oligosaccharides were similar to those recorded for the anologous *S. cerevisiae* X2180 *mnn9* mutant (11). All except the smallest gave mannose H-1 signals at δ4.77 (1 H), 4.87 (1 H), 5.03–5.06 (3 H), 5.09/5.11 (1 H), 5.125 (2 H), 5.29 (1 H), and 5.32 (1 H) that are characteristic of the $Man_{10}GlcNAc$ of the *mnn1 mnn9* strain, but they also showed various numbers of α1→3-linked mannose units at δ5.13–5.14. The smallest oligosaccharide (Fig. 3*A*) lacked one of the protons at δ5.03–5.06 and one at δ5.125, consistent with it having one less $\alpha Man\rightarrow^2\alpha Man\rightarrow^6$ unit.

Owing to limited amounts of the neutral oligosaccharides, we used HPLC and exo-α1→2-mannosidase digestion to investigate the locations of the α1→3-linked mannose units. The smallest CPY neutral oligosaccharide gave a H-1 NMR spectrum (Fig. 3*A*) consistent with structure **IV**, in which an extra α1→3-linked mannose was attached either to mannose A_4 (structure **Va**) or mannose C_2 (**Vb**).



Structure **IV**

The exomannosidase is expected to remove one mannose (C_2) from structure **Va** or two mannoses (A_3 and A_4) from

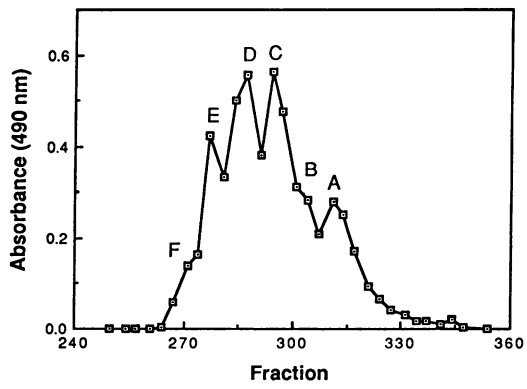


FIG. 2. Separation of neutral oligosaccharides by gel filtration. The oligosaccharide fraction from the Bio-Gel P-4 separation of the endoglucosaminidase H digest was applied to a Bio-Gel P-4 column (2 × 190 cm) and eluted with water. The effluent was monitored for total carbohydrate. The central tubes of the fractions were collected separately and labeled to correspond with the peaks in Fig. 1.

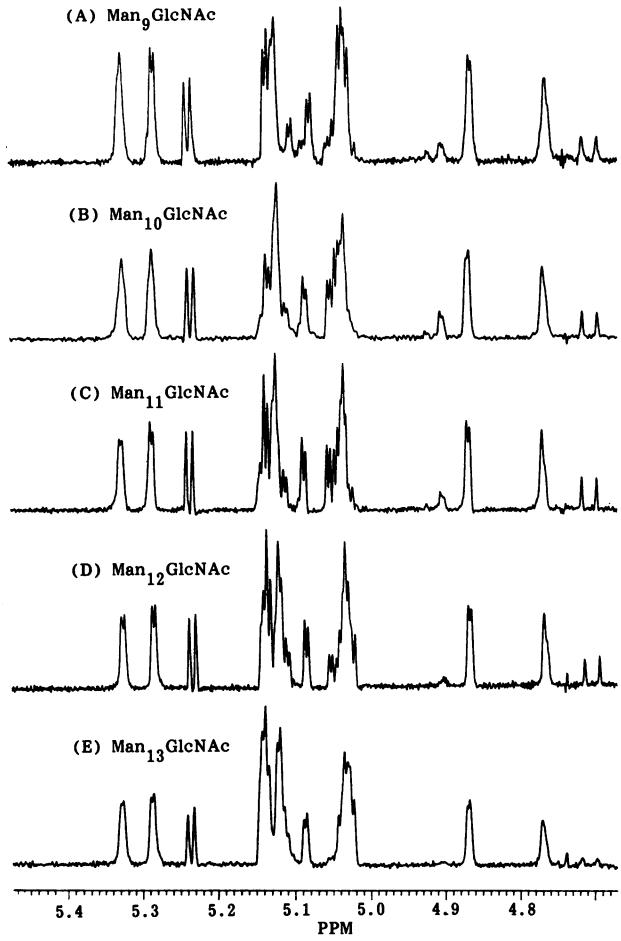
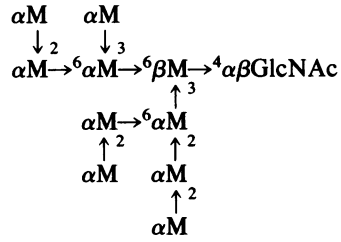


FIG. 3. H-1 NMR spectra of neutral oligosaccharides. (*A*) Peak A from Fig. 2 (a $Man_9GlcNAc$ with structure **Va**). (*B*) Peak B from Fig. 2 (a $Man_{10}GlcNAc$ with structure **VI** contaminated with the $Man_9GlcNAc$). (*C*) Peak C from Fig. 2 (a contaminated $Man_{11}GlcNAc$ with structure **VIIa**). (*D*) Peak D from Fig. 2 (a $Man_{12}GlcNAc$ with structure **VIIIa**). (*E*) Peak E from Fig. 2.

structure **Vb**. The HPLC pattern (Fig. 4) demonstrated that a single mannose was released because the resulting oligosaccharide was eluted at the position of a $Man_8GlcNAc$ (peak a). This established structure **Va** as the correct structure and is consistent with the reports that mannose A_4 is the first site of α1→3-mannosylation (11, 16).

Oligosaccharide B, with a retention time of $Man_{10}GlcNAc$, was converted to $Man_6GlcNAc$ (peak b) by exomannosidase digestion (Fig. 4). This change is consistent with structure **VI**, which lacks extra α1→3-linked mannose.



Structure **VI**

The similarity of the ^1H NMR spectrum (Fig. 3*B*) to that of the *mnn1 mnn9* $Man_{10}GlcNAc$ (8) confirmed this, although a weak signal at δ5.14 suggests contamination with oligosaccharide Va.

The $Man_{11}GlcNAc$ (Fig. 4, peak C) had a ^1H NMR spectrum (Fig. 3*C*) that was consistent with structure **VI**, in which an

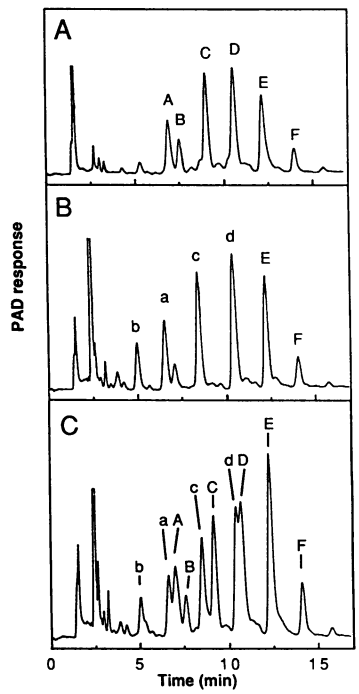


FIG. 4. HPLC patterns of oligosaccharides following digestion with exo-α1→2-mannosidase. The intact oligosaccharides are in uppercase letters, and the digestion products are in lowercase letters. (*A*) Undigested mixture. (*B*) Digested mixture. (*C*) Mixture of undigested and digested oligosaccharides: peak A, $Man_9GlcNAc$; peak B, $Man_{10}GlcNAc$; peak C, $Man_{11}GlcNAc$; peak D, $Man_{12}GlcNAc$; peak E, $Man_{13}GlcNAc$; peak F, $Man_{14}GlcNAc$; peak a, $Man_8GlcNAc$; peak b, $Man_6GlcNAc$; peak c, $Man_9GlcNAc$; peak d, $Man_{11}GlcNAc$. Peaks E and F were unchanged by the digestion. The indicated changes were confirmed by digesting the pure isolated oligosaccharides in separate reactions. PAD, pulsed ampometric detector.

extra α1→3-mannose was attached either to A_4 (structure **VIIa**), C_2 (structure **VIIb**), or d_2 (structure **VIIc**). The exomannosidase released two mannoses and produced an oligosaccharide (peak c) with the retention time of a $Man_9GlcNAc$ (Fig. 4), which supports structure **VIIa** because the other two isomers would lose three mannoses each by such treatment.

The $Man_{12}GlcNAc$ (Fig. 4, peak D) lost a single mannose upon exomannosidase digestion, which suggests it is a derivative of structure **VI**, in which two extra α1→3-mannoses are linked to A_4 and C_2 (structure **VIIIa**) or A_4 and d_2 (structure **VIIIb**). Because the $Man_{12}GlcNAc$ from the *mnn2 mnn9* mutant is predominantly the isomer with structure **VIIIa** (11), we favor that structure for this CPY homolog. In agreement, the ^1H NMR spectrum (Fig. 3*D*) is identical with that for the *mnn2 mnn9* homolog (11). The $Man_{13}GlcNAc$ was unaffected by the exomannosidase (Fig. 4, peak E), so all three terminal α1→2-linked mannoses (A_4, C_2, and d_2) must be substituted as they are in the corresponding oligosaccharide from the *mnn2 mnn9* mutant (11). The ^1H NMR spectrum (Fig. 3*E*) supports this conclusion.

^1H NMR Spectra of Phosphorylated CPY Oligosaccharides. The monophosphate derivative (Fig. 5*A*) gives a ^1H spectrum that is essentially identical to that for the oligosaccharide monophosphate from the *mnn9* mutant (11). After mild acid hydrolysis to cleave the glycosyl phosphate bond, the spectrum (Fig. 5*B*) showed the expected loss of the signals in the region δ5.40–5.44 and the reduction in intensity of the signal at δ5.16. Mannose and mannobiose, in a molar ratio of 1:1.3, were released by this treatment. Alkaline phosphatase released ≈80% of the monoester phosphate, and the HPLC pattern (Fig. 1*C*) revealed a mixture of homologs, the reten-

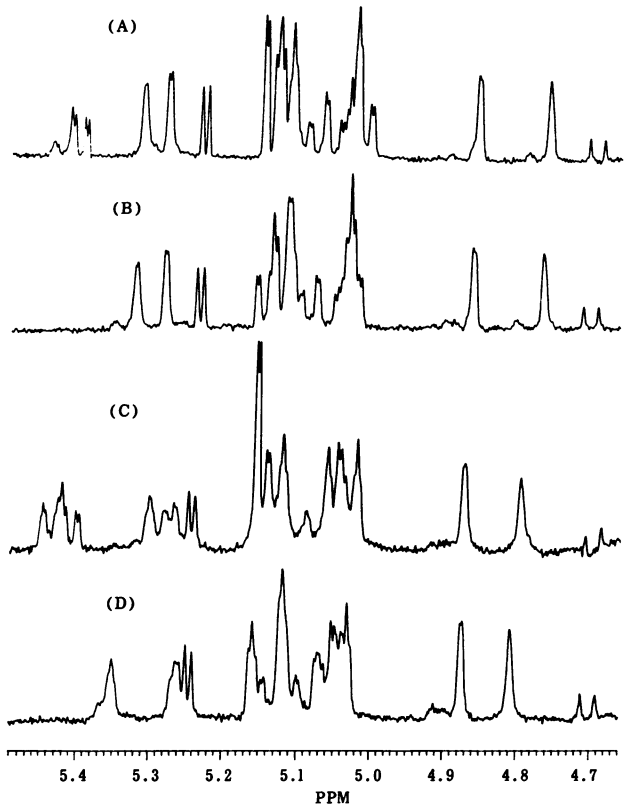


FIG. 5. H-1 NMR spectra of phosphorylated oligosaccharides: (*A*) monophosphate diester; (*B*) monoester formed by mild acid hydrolysis of monophosphate diester of *A*; (*C*) diphosphate diester; (*D*) monoester formed by mild acid hydrolysis of diphosphate diester of *C*.

tion times of which corresponded to those of the neutral oligosaccharides except that it lacked the $Man_9GlcNAc$ peak. Thus, this oligosaccharide apparently does not serve as a phosphate acceptor. Only 80% of the mild acid-hydrolyzed monophosphate fraction could be dephosphorylated by alkaline phosphatase.

The oligosaccharide diphosphate spectrum (Fig. 5*C*) shows the glycosyl phosphate signals as two overlapping doublets of doublets representing one mannosyl phosphate group and one mannobiosyl phosphate group. Hydrolysis of the glycosyl phosphate linkages causes a large downfield shift of H-1 for mannose A_2 to δ5.35, whereas mannose A_3 shifts further upfield to δ5.26. Attempted dephosphorylation of this product with alkaline or acid phosphatase led to removal of a single phosphate group, and the remaining oligosaccharide had the phosphate group on mannose A_3. Thus, the phosphate on mannose A_3 is unusually resistant to enzymic attack. The HPLC pattern of the oligosaccharide monophosphate revealed four homologs with retention times between 28 and 33 min under the conditions in Fig. 1 (12).

Jackbean α-Mannosidase Digestion of the Phosphorylated Oligosaccharides. To determine the locations of the phosphate groups, the mono- and diphosphate derivatives were digested with jackbean α-mannosidase, and the H-1 NMR spectra of the mannosidase-resistant phosphorylated fragments were determined. The monophosphate yielded a P-$Man_3GlcNAc$, as reported (7), but contrary to that report the spectrum (Fig. 6 *upper spectrum*) showed that the product consisted of a 4:1 mixture of structures **IX** and **X**.

$P\rightarrow{}^{6}\alpha Man\rightarrow{}^{6}\alpha Man\rightarrow{}^{6}\beta Man\rightarrow{}^{4}\alpha\beta GlcNAc$ Structure **IX**

$P\rightarrow{}^{6}\alpha Man\rightarrow{}^{2}\alpha Man\rightarrow{}^{3}\beta Man\rightarrow{}^{4}\alpha\beta GlcNAc$ Structure **X**

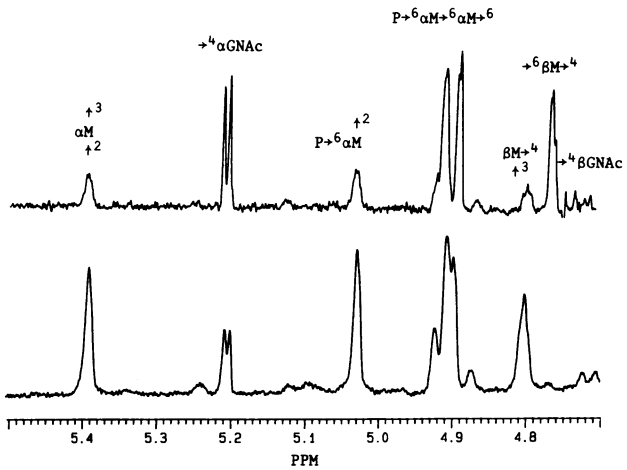


FIG. 6. H-1 NMR spectra of the phosphorylated products from jackbean α-mannosidase digestion of the oligosaccharide phosphate diesters. (*Upper spectrum*) P-$Man_3GlcNAc$ fragment from the oligosaccharide monophosphate; (*lower spectrum*) P_2-$Man_5GlcNAc$ fragment from the oligosaccharide diphosphate.

Structure **IX** results from the isomer phosphorylated at mannose C_1, and structure **X** results from the isomer phosphorylated at mannose A_3 (8, 10). Although substitution at position 3 is known to shield H-1 of the β-linked mannose, the phosphate on mannose A_3 has a dominant deshielding effect, so that H-1 of this mannose appears at δ4.80 instead of δ4.78. Note that the mannosidase removes mannose attached to phosphate as well as to other units, which accounts for the absence of signals at δ5.45. The isomer with the structure $P\rightarrow{}^{6}\alpha Man\rightarrow{}^{3}\alpha Man\rightarrow{}^{6}\beta Man\rightarrow{}^{4}\alpha\beta GlcNAc$ (7) would give a split H-1 signal at δ5.06/5.09 for the α1→3-linked mannose (8, 14), but no such signal was observed.

The ^1H spectrum of the mannosidase-resistant fragment from the diphosphate (Fig. 6 *lower spectrum*) was characteristic of a P_2-$Man_5GlcNAc$ derived from an oligosaccharide with phosphates on mannoses C_1 and A_3 (10) and of structure **XI**.

$$P\rightarrow{}^{6}\alpha M\rightarrow{}^{6}\alpha M\rightarrow{}^{6}\beta M\rightarrow{}^{4}\alpha\beta GlcNAc$$
$$\uparrow{}^{3}$$
$$\alpha M$$
$$\uparrow{}^{2}$$
$$\alpha M^{6}\leftarrow P$$

Structure **XI**

The β-linked mannose shows the downfield shift expected for phosphorylation on mannose A_3, and the integration of the signals at δ4.80 (1 H), 4.90–4.92 (2 H), 5.03 (1 H), and 5.39 (1 H) support this structure.

These results show that the oligosaccharide mono- and diphosphate consist predominantly of oligosaccharide **V** with a single mannosyl phosphate on C_2 (structure **XII**) or two mannosyl phosphates on C_2 and A_3 (structure **XIII**) with additional α1→3-linked mannoses being attached to the mannosyl phosphate units and mannoses A_4, C_2, and d_2. These are the same structures found in the *mnn2 mnn9* strain (11).

DISCUSSION

As pointed out (8), the $Man_{10}GlcNAc$ from the *mnn1 mnn2 mnn9 S. cerevisiae* mutant is phosphorylated on position 6 of mannose C_1, which precludes attachment of its outer chain to that position. Because a phosphate group in the wild-type CPY oligosaccharides is similarly located, the outer chain also must be attached elsewhere in these molecules. The mass spectra of the *mnn* mutant oligosaccharides and their

fragments place the outer chain on mannose A_2 (8), whereas the NMR spectra, HPLC analyses, and mannosidase digestions demonstrate the high probability that the CPY and *mnn2 mnn9* oligosaccharides are of the same structures. Our evidence that both positions of phosphorylation in the CPY and *mnn1 mnn9* oligosaccharides are also identical confirms this conclusion.

Enzymatic activities have been demonstrated for initiation of outer-chain synthesis (17), elongation (18), termination (19), branching (18), and phosphorylation (20). Consequently, we propose that enlargement of the $Man_8GlcNAc_2$-core oligosaccharide may occur as shown in Fig. 7. The pathway **A** → **B** → **C** → **D** outlines synthesis of the oligosaccharides found on CPY and on some sites on secreted invertase. This is the sole pathway expressed in the *mnn9* mutant. In a *mnn1 mnn9* strain, oligosaccharides with structure **C** are formed, whereas in the absence of *mnn1*, structure **C** is converted in three steps to structure **D** by an ordered addition of α1→3-linked mannoses to A_4, C_2, and d_2 (11, 16). Mannosyl phosphate units are added to C_1 and A_3 on oligosaccharide **C** and are also found at these sites in structure **D** (10), where they may be converted to mannobiosyl phosphate units (11), but whether phosphorylation can occur after addition of the α1→3-mannoses at C_2 and A_4 is not known (20).

The pathway **A** → **B** → **E** → **F** → **G** in Fig. 7 outlines synthesis of the larger oligosaccharides. In the *mnn1 mnn2 mnn10* mutant, oligosaccharides are produced with structure **F**, whereas a few have structure **E**, lacking the termination signal. In a *mnn2 mnn10* strain, α1→3-linked mannoses are added to A_4, C_2, and z_2, and mannosyl phosphates are found mainly on C_1 and z_1. Invertase molecules with an unbranched outer chain have been detected in a wild-type cell extract by probing with α1→6-mannosyl-specific antiserum (21).

In absence of the *mnn1* and *mnn2* lesions, large oligosaccharides are formed with structure **G**. The core and the terminus of the outer chain contain mannotriose units with the structure $\alpha M \rightarrow {}^3\alpha M \rightarrow {}^2\alpha M \rightarrow {}^6$, whereas the middle of the outer chain has tetrasaccharides with the structure $\alpha M \rightarrow {}^3\alpha M \rightarrow {}^2\alpha M \rightarrow {}^2\alpha M \rightarrow {}^6$. Some of the α1→6-linked mannose units of the outer chain are unsubstituted because acetolysis yields mannose in an amount exceeding that attributable to nonspecific degradation (22). The specificity of the mannosyl-phosphate transferase (20) suggests that the addition of these units to the side chains of the outer chain precedes addition of terminal α1→3-linked mannose units.

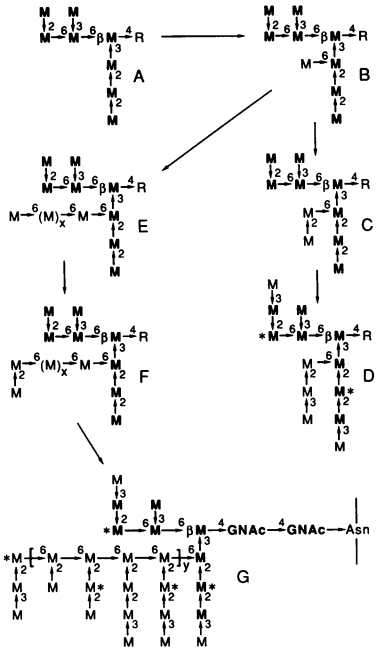


FIG. 7. Proposed pathway for enlargement of the $Man_8GlcNAc_2$ oligosaccharide on yeast mannoproteins. M, mannose; GNAc, *N*-acetylglucosamine. The value of x is 3–13 in the *mnn10* strain and 50–100 in the wild-type, whereas the value of y is 1–4 in the *mnn10* mutant and 10–25 in wild-type strains. Asterisks at mannoses in structures **D** and **G** indicate positions of phosphorylation, but phosphate is also found on the corresponding positions in structures **C** and **F**.

This pathway for mannoprotein glycosylation has several important features. The initiation, elongation, and termination reactions for outer-chain synthesis are structurally distinct and, presumably, subject to independent control. Although the termination step is superficially identical to the branching of the outer chain in that both involve α1→2-mannosylation of the α1→6-backbone, these processes are differentiated by the *mnn2* mutation, which affects branching but not termination. Although the termination step that leads to the small oligosaccharides found on CPY is tightly regulated in that it occurs after a single mannose addition, the elongated outer chain of the larger oligosaccharides in the *mnn10* mutant is variable in length (9), suggesting that this termination step is loosely controlled. This important branchpoint is defined by the *mnn9* mutation, which defect prevents elongation of the outer chain on all oligosaccharides. The recent report that plasma membrane receptor 1 (PMR1) mutants, which show ion pump and secretion defects, may also have a *mnn9*-like phenotype (23) is consistent with our view that the *mnn9* mutation affects the logistics of mannoprotein processing in the Golgi body.

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