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Characterization of Intermediates up to Lipid-Linked Heptasaccharide Implicated in the Biosynthesis of *Saccharomyces cerevisiae* Mannoproteins†

Chandra Prakash and Inder K. Vijay*

ABSTRACT: The lipid-linked oligosaccharide $Glc_3Man_9(GlcNAc)_2$ serves as a precursor for the biosynthesis of the inner core portion of the asparagine-linked polysaccharide of *Saccharomyces cerevisiae* mannoproteins. As a prelude to establishing its detailed structure and assembly, lipid-linked oligosaccharides belonging to the general structure $Man_n(GlcNAc)_2$, n = 1-5, and presumably serving as intermediates in the assembly sequence were isolated from an in vitro incubation of *S. cerevisiae* microsomes with UDP-*N*-acetylglucosamine and GDP-[^{14}C]mannose. On the basis of size, elution characteristics on a column of concanavalin A-Sepharose, exo- and endoglycosidase digestions, acetolysis, and methylation analysis, the major species within the tri- through heptasaccharides had the following structures: Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, Manα1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, Man(6←1αMan)α1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, Manα1→2Man(6←1αMan)α1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, and Manα1→2Manα1→2Man(6←1αMan)α1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc. These structures are identical with those of the major intermediates involved in the biosynthesis of asparagine-linked glycoproteins in animal tissues. Additionally, minor isomers were also observed in the tetra- through heptasaccharides and structurally characterized. The inner core of *S. cerevisiae* mannoproteins has been shown to have some structural differences from the high mannose glycoproteins of animal origin, notably in terms of terminally linked Manα1→3 residues in the side chains attached to the α1,6-linked polymannose backbone [Ballou, C. E. (1976) *Adv. Microb. Physiol. 14*, 93-158]. The initial studies reported here indicate that the lipid-linked assembly of the precursor unit for the inner core of *S. cerevisiae* mannoproteins might be similar to that in animal systems and modifications of the protein-linked polysaccharide occur that would give the final structure. The precise role of the minor isomers within the lipid-linked oligosaccharides in the assembly of the precursor oligosaccharide is presently unclear; it is possible that these arise due to a lack of specificity of the mannosyltransferases for acceptor substrates during the assembly process.

The mannoproteins of *Saccharomyces cerevisiae* consist of polymannose chains attached to both asparagine and hydroxy amino acid (serine and/or threonine) residues in the underlying polypeptides. The structure of the polysaccharide component of these glycoproteins, as proposed by Nakajima & Ballou (1974, 1975a,b), is shown in Figure 1. In this, about 10% of the mannose is attached to serine and threonine residues as short oligosaccharides of up to tetrasaccharide in size; the asparagine-linked polysaccharide, constituting nearly 90% of the total mannose, is present as two genetically differentiated sections. These are the inner core containing 12-17 mannose residues linked at the reducing end to an *N,N'*-diacetylchitobiose unit and a somewhat similar outer region of 150 or more mannose units. The entire structure is constructed on an α1,6-linked backbone with α1,2 and α1,3 linkages in the branches. The outer region also has occasional mannobiose phosphate side chains attached to the primary branches.

The biosynthesis of the inner core of the asparagine-linked polysaccharide of *S. cerevisiae* mannoproteins is somewhat analogous to the biosynthesis of similar units for animal glycoproteins (Lehle et al., 1980; Parodi, 1981; Trimble et al., 1980). A lipid (dolichol)-linked tetradecasaccharide, $Glc_3Man_9(GlcNAc)_2$,[1] is assembled within a membrane component of the cell and transferred en bloc to the polypeptide acceptors. This is followed by the excision of glucosyl residues by processing glucosidases (Lehle, 1980; Parodi, 1979a,b, 1981). At least one of the mannosyl residues of this newly transferred core portion also appears to be cleaved as it is elongated to build the outer region (Parodi, 1981). The mannose residues for the latter segment have been shown to be transferred directly from GDP-mannose to the inner core, catalyzed by several mannosyltransferases (Nakajima & Ballou, 1975b; Lehle, 1980; Parodi, 1979a,b). Additionally, a mannose phosphate transferase incorporates the phosphorylated mannose residues, which may be subsequently elongated by the addition of an α1,3-linked mannose unit (Karson & Ballou, 1978).

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[1] Abbreviations: DP, degree of polymerization; C/M, $CHCl_3$/CH_3OH; C/M/W, $CHCl_3/CH_3OH/H_2O$; CHO, Chinese hamster ovary; GlcNAc, *N*-acetylglucosamine; $(GlcNAc)_2$, *N,N'*-diacetylchitobiose; $GlcNAc_{OH}$, *N*-acetylglucosaminitol; Man, mannose; endo, endo-β-*N*-acetylglucosaminidase; Tris, tris(hydroxymethyl)aminomethane; Con A, concanavalin A.

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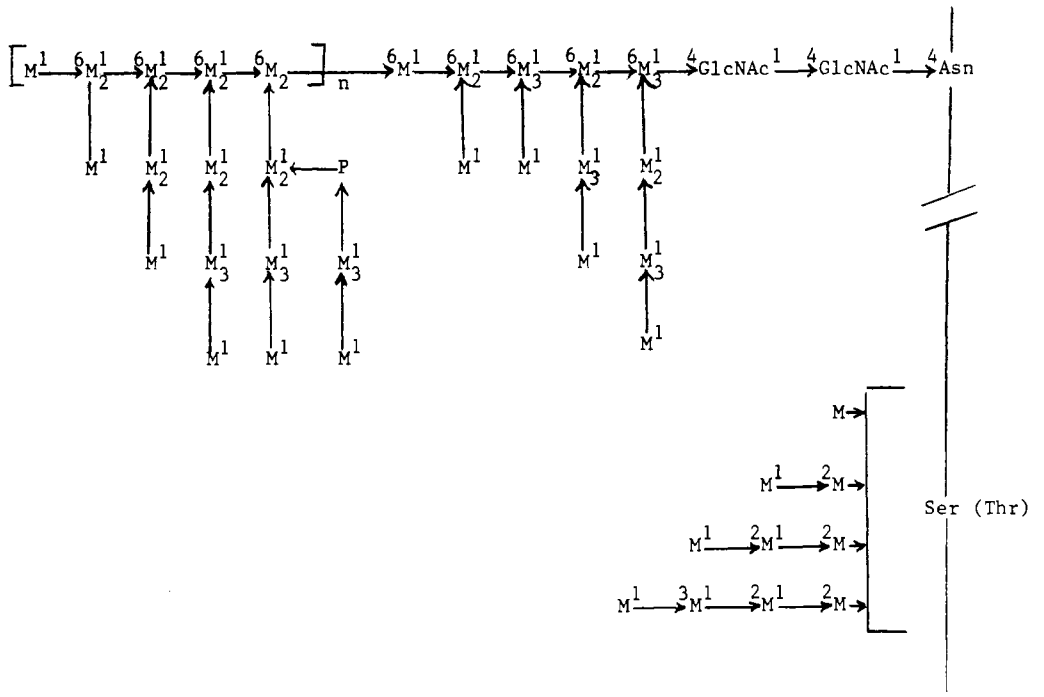

FIGURE 1: Carbohydrate portion of *S. cerevisiae* mannoprotein as proposed by Nakajima & Ballou (1975a). M stands for mannose and GlcNAc for *N*-acetylglucosamine. All the linkages are in α configuration except for that between the *N*-acetylglucosamine residues and that between the first mannose and the *N*-acetylglucosamine portion, which are β.

The detailed structure of the in vivo synthesized lipid-linked tetradecasaccharide $Glc_3Man_9(GlcNAc)_2$ precursor for the biosynthesis of asparagine-linked glycoproteins in Chinese hamster ovary (CHO) cells was shown to be Glc1→2Glc1→3Glc1→3Manα1→2Manα1→2Man[6←1αMan6←1αMan(3←1αMan2←1αMan)2←1αMan]α1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc (Li et al., 1978). Subsequently, an ordered sequence for the assembly of this structure was proposed by Chapman et al. (1979). Studies concerned with an in vitro sequence of lipid-linked reactions catalyzed by microsomes from the lactating bovine mammary tissue indicated that Man_1- to $Man_3(GlcNAc)_2$, $Man_9(GlcNAc)_2$ and the glucosyloligosaccharides Glc_1-, Glc_2-, and $Glc_3Man_9(GlcNAc)_2$ were monoisomeric (Vijay et al., 1980; Vijay & Perdew, 1980); however, two or more isomers within the Man_4- to $Man_8(GlcNAc)_2$ saccharides could be delineated (Vijay & Perdew, 1980; Perdew, 1981). The structures of all of the monoisomeric saccharides and the major isomers within the multiisomeric intermediates were identical with the structures proposed for the in vivo assembly in CHO cells. A supplementation of the in vitro incubations of the bovine mammary tissue reduced the relative proportions and the number of isomers within the Man_4- to $Man_8(GlcNAc)_2$ oligosaccharides linked to the lipid carrier in favor of the ordered sequence proposed for the in vivo assembly of the oligosaccharide precursor in CHO cells.

The structure of the inner core of *S. cerevisiae* reveals certain differences from the interresidue linkages within the oligosaccharide portions of high mannose glycoproteins of animals (Kornfeld & Kornfeld, 1980) as well as the linkages within their precursor oligosaccharide, $Glc_3Man_9(GlcNAc)_2$, linked to the lipid carrier (Li et al., 1978). This has brought several questions into focus: (i) Is the stepwise lipid-linked assembly of the $Glc_3Man_9(GlcNAc)_2$ unit, as proposed for the major biosynthetic sequence in animal cells, operative in all eukaryotes including the fungi? (ii) Do multiple pathways exist for the biosynthesis of the core portion of the yeast mannan that might give a basis for its microheterogeneity? (iii) Is more than one mannose residue trimmed off from the newly protein-attached core prior to the incorporation of α1,3-linked mannose residues at the nonreducing termini of the branches in the core? (iv) What is the time frame of mannosyl trimming of the core in relation to the elongation reactions for the construction of the outer region? We have isolated and conducted a structural analysis on the lipid-linked oligosaccharides up to heptasaccharides from incubations of *S. cerevisiae* microsomes with UDP-*N*-acetylglucosamine and GDP-mannose. Our results show that the initial portion of the major sequence for the assembly of the lipid-linked oligosaccharides in the yeast appears to be similar to the one proposed for animal systems (Chapman et al., 1979; Vijay et al., 1980; Vijay & Perdew, 1980). Further, several of the oligosaccharides have multiple isomers. It is possible that the minor isomers within the oligosaccharides arise from lack of absolute substrate specificity of mannosyltransferases involved in the assembly process.[2]

Experimental Procedures

Materials. GDP-[^{14}C]mannose (192 mCi/mmol) and NaB^3H_4 (220 mCi/mmol) were purchased from New England Nuclear. UDP-*N*-acetylglucosamine, jack bean α-mannosidase, β-glucuronidase, and concanavalin A–Sepharose were the products of Sigma. Endo-β-*N*-acetylglucosaminidases D and H were obtained from Miles Laboratories and checked for substrate specificity and any cross contamination with interfering enzymes by using the [^{14}C]mannose-labeled substrates Man(6←1αMan)α1→3Manβ1→4(3)GlcNAcB1→4(3)GlcNAc [$Man_3(GlcNAc)_2$] and Manα1→2Manα1→2Man[6←1αMan6←1αMan(3←1αMan2←1αMan)2←1αMan]α1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc [$Man_9(GlcNAc)_2$], isolated and characterized earlier (Vijay et al., 1980). β-Mannosidase was purified from *Polyporus sulfureus* (Wan et al., 1976). The following enzymes were kindly donated by the individuals noted: homogeneous

[2] A preliminary account of this work has appeared (Prakash & Vijay, 1981).

α1,2-specific mannosidase from *Aspergillus saitoi* (Yamashita et al., 1980, Ichishima et al., 1981) (Dr. E. Ichishima, Tokyo Noko University) and endo-β-*N*-acetylglucosaminidase L (Trimble et al., 1979) (Drs. R. B. Trimble and F. Maley, New York Department of Health). The α1,2-mannosidase was free from α1,3- and α1,6-mannosidase activity since it failed to release [^{14}C]mannose from the labeled $Man_3(GlcNAc)_2$ given above and gave [^{14}C]mannose and a labeled product that chromatographed with the $Man_3(GlcNAc)_2$ standard when incubated with the [^{14}C]mannose-labeled $Man_9(GlcNAc)_2$ given above. The oligosaccharides Man_1- through $Man_9(GlcNAc)_2$ were standards from our previous studies (Vijay et al., 1980).

Organism. The following *S. cerevisiae* strains were used: wild type, X-2180-1B(α) (obtained from Yeast Genetics Stock Center, University of California, Berkeley, CA), and ATCC 24297 (isolated from Fleishmann's brand of bakers' yeast). The partially O-methylated mannose standards were from laboratory collection. [^{14}C]Mannose-labeled Manβ1→4(3)-$GlcNAc_{OH}$, Manα1→3Manβ1→4(3)$GlcNAc_{OH}$, and Manα1→2Manα1→3Manα1→3Manβ1→4(3)$GlcNAc_{OH}$ were prepared as described before (Vijay et al., 1980).

Membrane Fraction. The *S. cerevisiae* strains were grown at 25 °C to early logarithmic phase in 2% glucose, 0.5% yeast extract, and 2% Bacto peptone. The particulate enzyme fraction was prepared according to the procedure of Lehle (1980).

Preparation of Lipid-Linked Saccharides. The lipid-linked oligosaccharides were prepared as follows: The membrane fraction, 300 mg of protein, 40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 8 mM $MgCl_2$, and 0.1 mM UDP-GlcNAc and 12 μCi of GDP-[^{14}C]mannose in a total volume of 30 mL were incubated at 37 °C for 20 min. The reaction was terminated by adding the entire incubation mixture into 600 mL of C/M, 2:1. Further processing of the reaction mixture, followed by mild acid hydrolysis, ion exchange, and gel filtration of the oligosaccharides, was essentially the same as before (Vijay et al., 1980), including the step in which all saccharides except the radioactive mannose from the Bio-Gel P-4 column were collected for lyophilization prior to paper chromatography.

Glycosidase Digestions. The conditions for the digestion of oligosaccharides with different exo- and endoglycosidases have been given before (Vijay et al., 1980). For digestion with α1,2-mannosidase, the individual oligosaccharides were incubated with 200 ng of the enzyme in 0.05 mL of 1 M sodium acetate buffer, pH 5.0, under a toluene atmosphere at 37 °C. After 24 h, an additional 200 ng of the enzyme was added and the incubation continued for a total of 48 h. The digest was subsequently deproteinized and desalted as for other glycosidase digestions in preparation for paper chromatography.

Chromatographic Procedures. For affinity chromatography, a column (0.5 × 5 cm) of concanavalin A–Sepharose was equilibrated with 0.01 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl (Ogata et al., 1975). Radioactive oligosaccharides in 0.1 mL of water were applied to the column, and the column was washed with 10 mL of the above buffer. Fractions of 1 mL were collected. The column was then eluted with 10 mL of 0.1 M methyl α-mannoside in the above buffer, and 1-mL fractions were collected. Radioactivity in the fractions was determined by scinitillation counting in ScintiVerse (Fisher Chemical Co.).

Descending paper chromatography on Schleischer and Schüll paper 589 C was performed with ethyl acetate/pyridine/acetic acid/water, 5:5:1:3 (solvent A), and butanol/pyridine/water, 4:3:4 (solvent B). Usually, paper chromatograms were developed for 30–33 h in solvent A and for 88 h in solvent B. Thin-layer plates of silica gel G (EM Labs) were developed in benzene/acetone/water/NH_4OH, 50:200:3:1.5 (solvent C). One-centimeter sections from paper chromatograms and 0.5-cm scrapings from thin-layer chromatograms were counted by liquid scintillation.

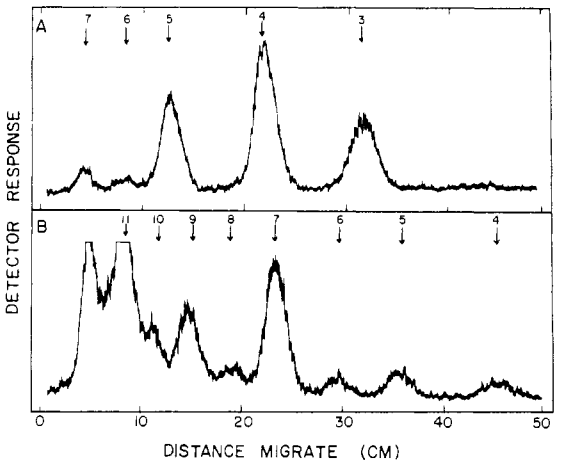

FIGURE 2: Paper chromatograms of [^{14}C]mannose-labeled oligosaccharides obtained from C/M, 2:1, and C/M/W, 10:10:3, soluble glycolipids extracted from incubation of *S. cerevisiae* microsomes with GDP-[^{14}C]mannose as given under Experimental Procedures. (Panel A) Oligosaccharides obtained from the C/M, 2:1, extract were chromatographed in solvent A; (panel B) oligosaccharides obtained from the C/M/W, 10:10:3, extract were chromatographed in solvent B. The arrows indicate the migrations of standards: (3) Man-$(GlcNAc)_2$, (4) $Man_2(GlcNAc)_2$, (5) $Man_3(GlcNAc)_2$, (6) Man_4-$(GlcNAc)_2$, (7) $Man_5(GlcNAc)_2$, (8) $Man_6(GlcNAc)_2$, (9) Man_7-$(GlcNAc)_2$, (10) $Man_8(GlcNAc)_2$, and (11) $Man_9(GlcNAc)_2$.

Structural Methods. Details of other techniques including gel filtration on a calibrated column of Bio-Gel P-4, methylation analysis, and acetolysis have been described previously (Vijay et al., 1980). Protein was determined by the Lowry procedure (Lowry et al., 1951).

Results

Isolation of Labeled Saccharides. The [^{14}C]mannose-labeled oligosaccharides obtained after mild acid hydrolysis of the lipid-linked oligosaccharides, as given under Experimental Procedures, were subjected to paper chromatography in solvents A and B. A heterogeneous mixture of oligosaccharides could be resolved (Figure 2). On the basis of comparison with standards, the oligosaccharides obtained from the C/M, 2:1, extract appeared to be Man_1- to $Man_5(GlcNAc)_2$, whereas the oligosaccharides obtained from the C/M/W, 10:10:3, extract were most likely Man_2- to $Man_9(GlcNAc)_2$ and higher oligosaccharides. This is typical of the profile of oligosaccharides obtained from similar studies with animal systems (Vijay et al., 1980; Rearick et al., 1981). For the structural studies reported here, a detailed characterization of the oligosaccharides up to a DP of 7 was conducted.[3] The radioactivity under peaks III–VII was eluted from the paper, and individual oligosaccharides were further purified by rechromatography

[3] The solubility characteristics of lipid-linked tetra- through heptasaccharides are such that these are partially extracted into C/M, 2:1, and the remainder into C/M/W, 10:10:3, during multiple solvent extraction. However, the water-soluble oligosaccharides obtained after mild acid hydrolysis of these glycolipids soluble in the two solvent mixtures are indistinguishable by the usual chromatographic procedures employed. Thus, the tetra- through heptasaccharides obtained from glycolipids soluble in C/M/W, 10:10:3, were combined with the corresponding oligosaccharides from glycolipids soluble in C/M, 2:1, and analyzed together.

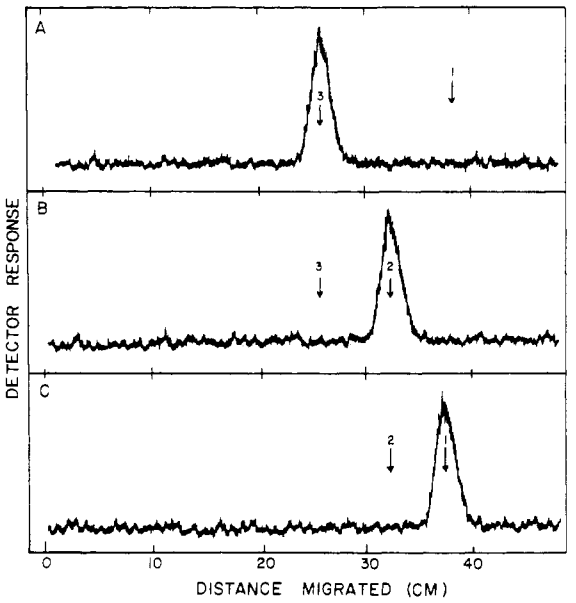

FIGURE 3: Digestion of [^{14}C]mannose-labeled saccharide III with α-mannosidase (panel A) and endo L (panel B). The radioactive peak in panel B was further digested with β-mannosidase (panel C). The paper chromatograms were developed in solvent A. The arrows indicate the positions of standards: (1) mannose, (2) Manβ1→4-(3)GlcNAc, and (3) Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc.

on the paper in solvent A and by gel filtration on a calibrated column of Bio-Gel P-4 (2 × 189 cm, −400 mesh; Vijay et al., 1980).

Essentially identical results were obtained for the structural analysis and relative ratios of isomers within the oligosaccharides obtained from microsomal incubations of *S. cerevisiae* X-2180-1B(α) and ATCC 24297. For avoidance of duplication, all the results given below, with one exception, are on the lipid-linked oligosaccharides prepared from strain X-2180-1B(α). The exception was the heptasaccharide; while only one isomer of this intermediate was synthesized by strain X-2180-1B(α), strain ATCC 24297 also synthesized an additional minor isomer for which the data are provided below.

Structural Characterization of Saccharide III. (i) It was totally resistant to the action of α-mannosidase (Figure 3A). (ii) Upon treatment with endo L, it yielded a labeled disaccharide that cochromatographed with the Manβ1→4(3)-GlcNAc standard (Figure 3B). (iii) The disaccharide from (ii) gave [^{14}C]mannose as product upon digestion with β-mannosidase (Figure 3C). (iv) It was resistant to acetolysis, a technique that preferentially cleaves α1,6 linkages in oligosaccharides, and digestion by endo D and endo H.

Trimble et al. (1979) have shown that endo L hydrolyzes the bond between the two *N*-acetylglucosamine residues at the reducing terminus of chitobiosyloligosaccharides. The enzyme will also hydrolyze, albeit at a reduced rate, a similar linkage of Man(GlcNAc)$_2$ and Man(GlcNAc)$_2$Asn substrates. Any additional mannosylation of these compounds totally abolishes their susceptibility to cleavage by this enzyme. These results are consistent with the structure of the trisaccharide being Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc.

Structural Characterization of Saccharide IV. (i) When digested with α-mannosidase, it released [^{14}C]mannose and a labeled trisaccharide that cochromatographed with Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc (Figure 4A). Because of the endogenous lipid-linked oligosaccharides in the microsomes, the labeling of mannose residues in the oligosaccharide is not uniform. (ii) The trisaccharide from Figure 4A had all the characteristics of lipid-linked saccharide III.

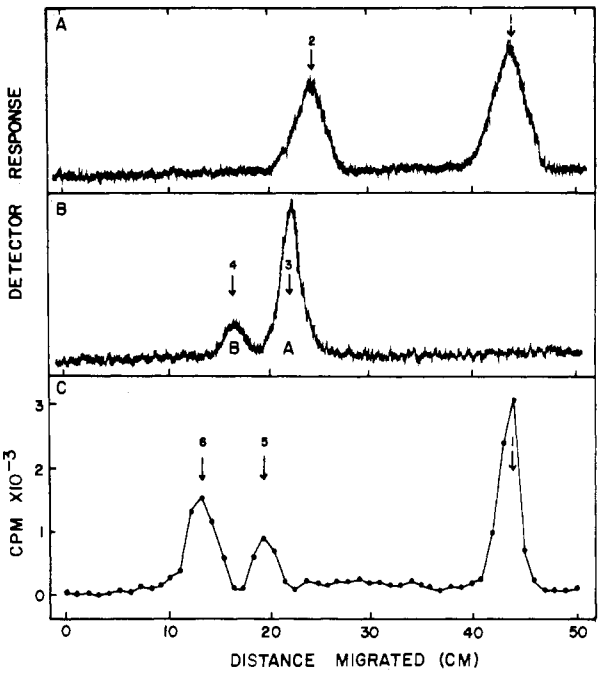

FIGURE 4: Structural studies on [^{14}C]mannose-labeled saccharide IV: (panel A) treatment of saccharide IV with α-mannosidase; (panel B) digestion of saccharide IV with endo D; (panel C) acetolysis of $NaBH_4$-reduced, endo D resistant saccharide IV (peak B, panel B). The paper chromatograms were developed in solvent A. The arrows indicate the positions of standards: (1) mannose, (2) Manβ1→4-(3)GlcNAcβ1→4(3)GlcNAc, (3) Manα1→3Manβ1→4(3)GlcNAc, (4) saccharide IV, (5) Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc$_{OH}$, and (6) Manα1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc$_{OH}$.

(iii) Upon digestion with endo D, nearly 85% of saccharide IV was cleaved to give a product that comigrated with Manα1→3Manβ1→4(3)GlcNAc standard (Figure 4B). This enzyme cleaves the *N,N'*-diacetylchitobiose linkage in oligo-(mannosylchitobiosyl)saccharides of the general structure Man$_n$(3←1αMan)α→Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, $n \geq 0$ (Tai et al., 1975, 1977). (iv) The oligosaccharide fragment obtained after cleavage of saccharide IV with endo D and presumably Man$_2$GlcNAc was reduced with $NaBH_4$ and subjected to acetolysis; it was totally resistant to acetolysis, indicating the absence of any α1,6 linkages in this fragment. (v) The endo D resistant saccharide IV was also reduced with $NaBH_4$ and subjected to acetolysis. It gave [^{14}C]mannose and a labeled trisaccharide that had the mobility of the Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc$_{OH}$ standard (Figure 4C). The conditions of the acetolysis reaction for this and subsequent experiments were chosen to give a minimum degree of overdegradation. A reacetolysis of the undegraded starting material always generated a fragmentation pattern that was identical with the original one. (vi) An analysis of endo D susceptible saccharide IV, examined by the methylation of the Man$_2$GlcNAc fragment in (ii), gave 2,4,6-tri-*O*-methyl- and 2,3,4,6-tetra-*O*-methylmannose as products (Figure 5A). However, endo D resistant tetrasaccharide gave 2,3,4-tri-*O*-methyl-and 2,3,4,6-tetra-*O*-methylmannose as products (Figure 5B). On the basis of these results, the following structures may be proposed for the two isomers in saccharide IV: Manα1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc (saccharide IV-1, 85%) and Manα1→6Manβ1→4(3)GlcNAcβ1→4-(3)GlcNAc (saccharide IV-2, 15%).

Structural Characterization of Saccharide V. (i) Upon treatment with α-mannosidase, it yielded [^{14}C]mannose and a labeled trisaccharide that comigrated with Manβ1→4(3)-GlcNAcβ1→4(3)GlcNAc (Figure 6A). (ii) The trisaccharide

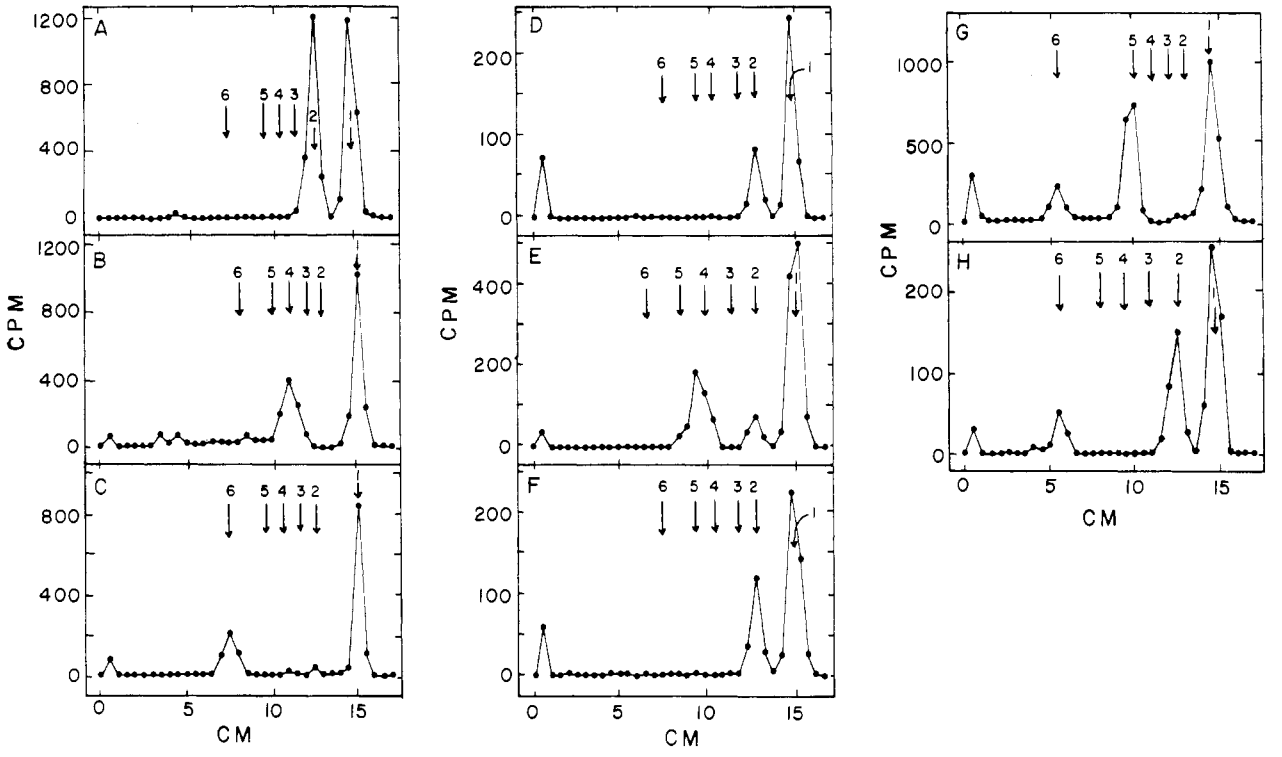

FIGURE 5: Methylation analysis of [^{14}C]mannose-labeled oligosaccharides and their fragments obtained after enzymatic and acetolysis degradations: (panel A) $Man_2GlcNAc$ derived from the action of endo D on saccharide IV; (panel B) endo D resistant saccharide IV; (panel C) $Man_3GlcNAc$ derived from the action of endo D on saccharide V; (panel D) endo D and α1,2-mannosidase-resistant saccharide V; (panel E) fragment corresponding to $Man_3GlcNAcGlcNAc_{OH}$, obtained after acetolysis of saccharide VI; (panel F) α1,2-mannosidase-resistant saccharide VI; (panel G) saccharide VII eluted from Con A–Sepharose column with 0.1 M methyl α-mannoside; (panel H) α1,2-mannosidase-resistant heptasaccharide from *S. cerevisiae* strain ATCC 24297. Silica gel G plates were developed 2 times in solvent C. The arrows indicate the positions of standards: (1) 2,3,4,6-tetra-*O*-MeMan, (2) 2,4,6-tri-*O*-MeMan, (3) 2,3,6-tri-*O*-MeMan, (4) 2,3,4-tri-*O*-MeMan, (5) 3,4,6-tri-*O*-MeMan, and (6) 2,4-di-*O*-MeMan.

fragment had all the characteristics of saccharide III given above. (iii) Nearly 90% of saccharide V was susceptible to cleavage by endo D (Figure 6B). (iv) Fragment B in Figure 6B was reduced with $NaBH_4$ and subjected to acetolysis (Figure 6C). It released mannose and $Man_2GlcNAc_{OH}$ as products. (v) When examined by affinity chromatography on a column of Con A–Sepharose, ~10% of saccharide V was recovered in the elution buffer and about 90% was bound and eluted with 100 mM methyl α-mannoside (Figure 7A). The binding of oligosaccharide to Con A–Sepharose requires at least two α-linked mannose residues in its structure; further, the hydroxyl groups at positions 3, 4, and 6 should be free (Ogata et al., 1975). (vi) A methylation analysis of peak B in Figure 4B gave 2,4-di-*O*-methyl- and 2,3,4,6-tetra-*O*-methylmannose (Figure 5C). On the basis of the results above, the major isomer in saccharide V has the structure Man(3←1αMan)α1→6Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc (saccharide V-1).

The endo D resistant pentasaccharide represented by peak A in Figure 4B was totally resistant to acetolysis. Upon treatment with α1,2-specific mannosidase, 64% of the radioactivity in this fraction was resistant to digestion whereas the remainder was cleaved to give [^{14}C]mannose and a radioactive fragment that migrated as $Man_2(GlcNAc)_2$ (Figure 6D). A further treatment of the radioactivity under peak B in Figure 6D with endo D gave a radioactive fragment that had the chromatographic chracteristics of $Man_2GlcNAc$ (Figure 6E). Since Manα1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc is the smallest oligosaccharide that is compatible with the specificity requirements for digestion by endo D (Vijay & Perdew, 1980), the radioactive fragment in peak B (Figure 6D) must have this structure. Thus, 36% of the endo D resistant saccharide V or 3.6% of the total saccharide V fraction represents a pentasaccharide that most likely has the structure Manα1→2Manα1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc (saccharide V-3).

A methylation analysis of the endo D as well as α1,2-mannosidase-resistant pentasaccharide yielded radioactive peaks corresponding to 2,4,6-tri-*O*-methyl- and 2,3,4,6-tetra-*O*-methylmannose (Figure 5D). The only structure compatible with these results is the pentasaccharide Manα1→3Manα1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc (saccharide V-2). This isomer represents about 6.4% of the total lipid-linked pentasaccharide (saccharide V-2).

The above results show that saccharide V consists of three isomers. Among these, the major isomer is saccharide V-1. This is also the intermediate in the major pathway of lipid-linked assembly of precursor oligosaccharide for the biosynthesis of asparagine-linked glycoproteins in animal tissues (Chapman et al., 1979; Vijay et al., 1980). The significance of saccharides V-2 and V-3 for the biosynthesis of mannoproteins in *S. cerevisiae* is given under Discussion.

Structural Characterization of Saccharide VI. (i) Treatment of saccharide VI with α-mannosidase yielded [^{14}C]-mannose and a radioactive trisaccharide that cochromatographed with the Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc standard (Figure 8A). (ii) The trisaccharide had all the structural features of saccharide III, given before. (iii) Saccharide VI was totally resistant to cleavage by either endo D or endo H (not shown). The specificity of endo D enzyme toward oligosaccharide substrates was mentioned earlier. Endo H cleaves the *N,N'*-diacetylchitobiosyl linkage in oligo(mannosylchitobiosyl)saccharides commonly found in high mannose glycoproteins as long as a mannose residue is linked α1,3 to

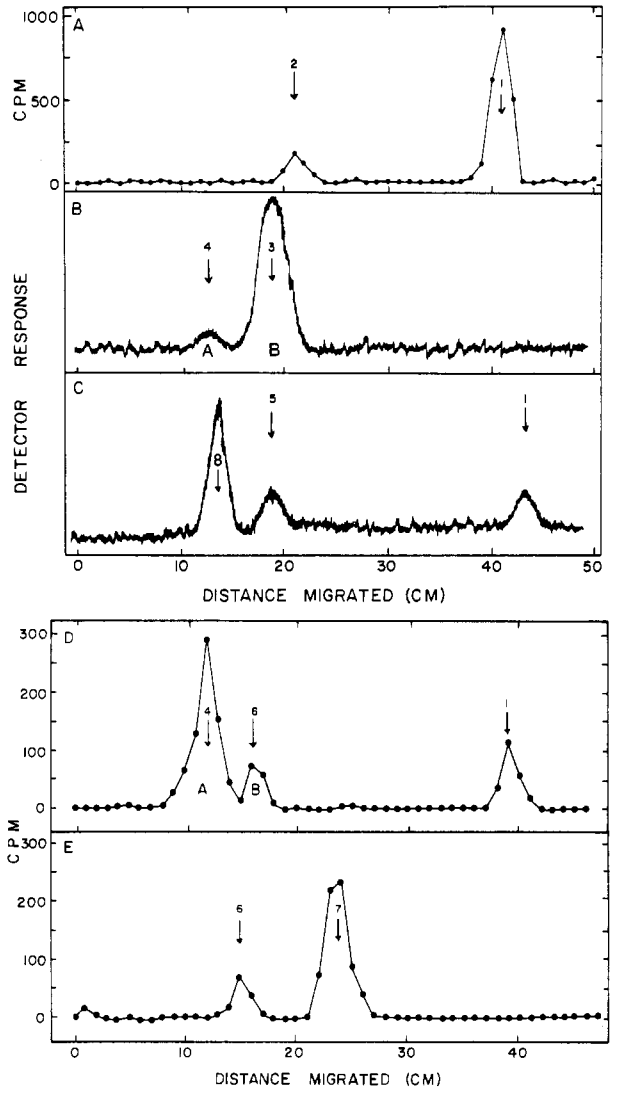

FIGURE 6: Structural studies on [^{14}C]mannose-labeled saccharide V: (panel A) digestion with α-mannosidase; (panel B) digestion with endo D; (panel C) acetolysis of $NaBH_4$-reduced radioactive peak B in panel B; (panel D) α1,2-mannosidase digestion of radioactivity in peak A of panel B; (panel E) treatment of radioactive peak B in panel D with endo D. The paper chromatograms were developed in solvent A. The arrows indicate the positions of standards: (1) mannose, (2) Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, (3) Man(6← 1αMan)α1→3Manβ1→4(3)GlcNAc, (4) saccharide V, (5) Manα1→3Manβ1→4GlcNAc$_{OH}$, (6) Manα1→3Manβ1→4(3)-GlcNAcβ1→4(3)GlcNAc, (7) Manα1→3Manβ1→4(3)GlcNAc, and (8) Man(6←1αMan)α1→3Manβ1→4(3)GlcNAc$_{OH}$.

the mannose residue that is further linked α1,6 to the β mannose attached to the *N*,*N*′-diacetylchitobiose (Tai et al., 1977; Vijay & Perdew, 1980). (iv) Upon digestion with α1,2-mannosidase, nearly 80% of the radioactivity in saccharide VI was degraded to [^{14}C]mannose and a product that cochromatographed with the pentasaccharide Man(6← 1αMan)α1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc (Figure 8B). (v) The pentasaccharide fragment from (iv) was susceptible to cleavage by endo D and gave $Man_3GlcNAc$ as the product (Figure 8C). (vi) Acetolysis of $NaBH_4$-reduced saccharide VI yielded [^{14}C]mannose, a fragment that appeared to be $Man_3GlcNAcGlcNAc_{OH}$, and undegraded saccharide VI (Figure 8D). (vii) A methylation analysis of the $Man_3GlcNAcGlcNAc_{OH}$ fragment from (vi) gave 2,4,6-tri-*O*-methyl-, 3,4,6-tri-*O*-methyl, and 2,3,4,6-tetra-*O*-methyl-mannose as products (Figure 5E). (viii) When chromatographed on a column of Con A–Sepharose, 22% of saccharide

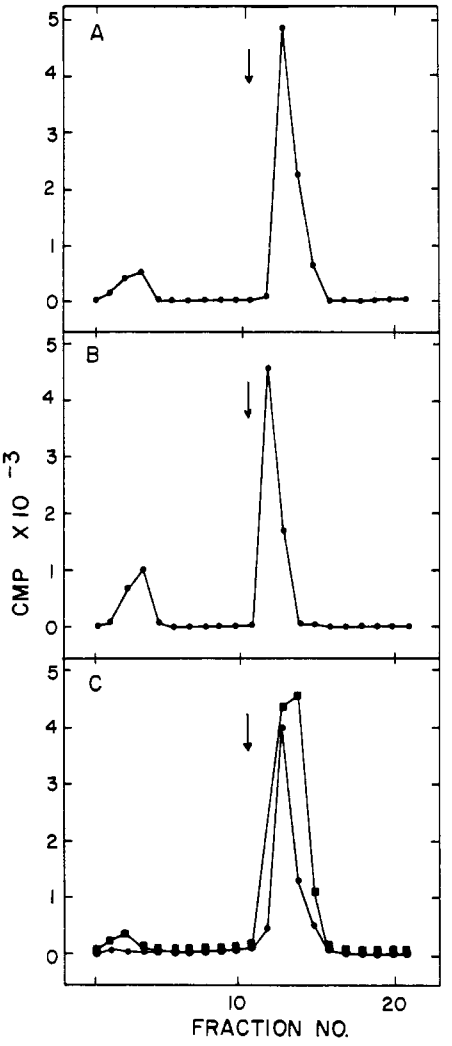

FIGURE 7: Concanavalin A–Sepharose chromatography of saccharides V, VI, and VII. Details of the experiment are given in the text. The arrow indicates the position at which 100 mM methyl α-mannoside was included in the equiliberation buffer for elution. (panel A) Elution of saccharide V; (panel B) elution of saccharide VI; (panel C) elution of saccharide VII from strain X-2180-1B(α) (●) and saccharide VII from strain ATCC 24297 (■).

VI did not bind to the column whereas 78% was bound and eluted with 100 mM methyl α-mannoside (Figure 7B). Saccharide VI that did not bind to the Con A–Sepharose column was totally resistant to the action of α1,2-mannosidase. This agrees with the results given under (iv). All of the results given above are consistent with the following hexasaccharide as the major isomer in saccharide VI: Manα1→2Man(6← 1αMan)α1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc (saccharide VI-1).

The minor isomer constituting ~20–22% of saccharide VI and resistant to digestion by α1,2-mannosidase, endo D, and endo H (see above) was also resistant to acetolysis (not shown). Upon methylation it gave 2,4,6-tri-*O*-methyl- and 2,3,4,6-tetra-*O*-methylmannose as products (Figure 5F). The only structure that satisfies these criteria is the linear oligosaccharide Manα1→3Manα1→3Manα1→3Manβ1→4(3)-GlcNAcβ1→4(3)GlcNAc (saccharide VI-2). Since the hydroxyl groups at C-3 of α-mannose residues in this structure are linked in glycosidic bonds, it would not be expected to bind to Con A (Ogata et al., 1975). Saccharide VI-2 is most likely derived by incorporation of an α1,3-linked mannose at the nonreducing end of saccharide V-2.

The major isomer in the hexasaccharide, i.e., saccharide

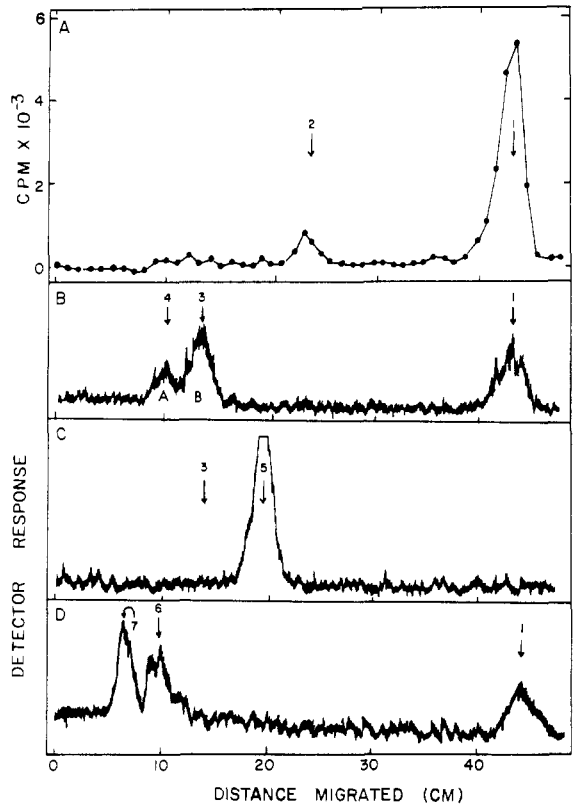

FIGURE 8: Structural studies on saccharide VI: (panel A) digestion with α-mannosidase; (panel B) digestion with α1,2-mannosidase; (panel C) treatment of peak B of panel B with endo D; (panel D) acetolysis of $NaBH_4$-reduced saccharide VI. The paper chromatograms were developed in solvent A. The arrows indicate the positions of standards: (1) mannose, (2) Manβ1→4(3)GlcNAcβ1→4(3)-GlcNAc, (3) Man(6←1αMan)α1→3Manβ1→4(3)GlcNAcβ1→4-(3)GlcNAc, (4) saccharide VI, (5) Man(6←1αMan)α1→-3Manβ1→4(3)GlcNAc, (6) Man(6←1αMan)α1→3Manβ1→4(3)-GlcNAcGlcNAc$_{OH}$, and (7) undegraded $NaBH_4$-reduced saccharide VI.

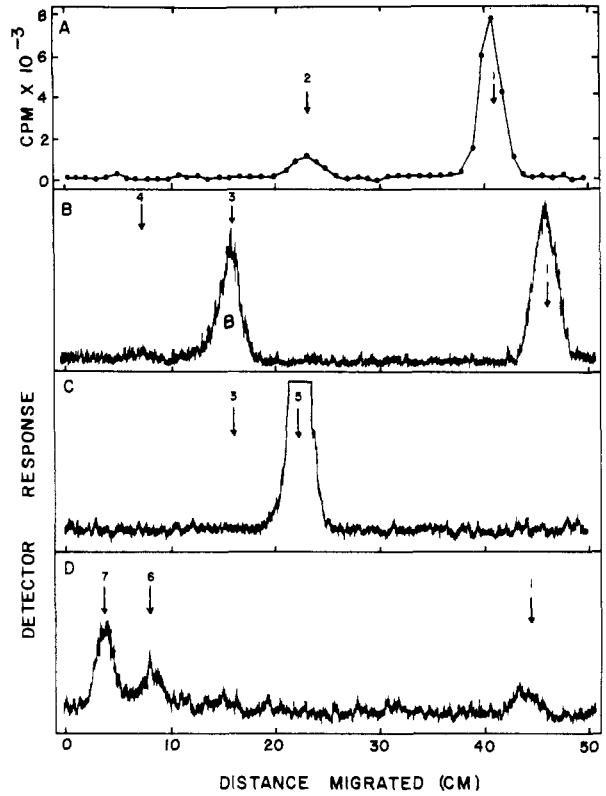

FIGURE 9: Structural studies on saccharide VII: (panel A) digestion with α-mannosidase; (panel B) digestion of saccharide VII isolated from lipid-linked saccharides synthesized by microsomes from strain 24297 of *S. cerevisiae* with α1,2-mannosidase; (panel C) treatment of peak B of panel B with endo D; (panel D) acetolysis of $NaBH_4$-reduced saccharide VII. The paper chromatograms were developed in solvent A. The arrows indicate the positions of standards: (1) mannose, (2) Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, (3) Man(6←1αMan)α1→3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc, (4) intact saccharide VII, (5) Man(6←1αMan)α1→3Manβ1→4(3)-GlcNAc, (6) Manα1→2Manα1→2Manα1→3Manβ1→4(3)-GlcNAcβ1→4(3)GlcNAc$_{OH}$, and (7) $NaBH_4$-reduced saccharide VII.

VI-1, is the same as the predominant hexasaccharide synthesized by the lipid-linked assembly sequence in animal systems (Chapman et al., 1979; Vijay & Perdew, 1980).

Structural Characterization of Saccharide VII. (i) Upon digestion with α-mannosidase, it released [^{14}C]mannose and a labeled trisaccharide that cochromatographed with the Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc standard (Figure 9A). (ii) The trisaccharide had all the characteristics of saccharide III. (iii) Saccharide VII was totally resistant to the action of both endo D and endo H. (iv) The heptasaccharide isolated from microsomal incubation of strain X-2180-1β(a) was completely digested by α1,2-mannosidase and gave [^{14}C]-mannose and a pentasaccharide as product (not shown). However, ~7% of the heptasaccharide isolated from microsomal incubation of strain ATCC 24297 was resistant to the action of α1,2-mannosidase, whereas the remaining 93% was cleaved similar to the heptasaccharide from strain X-2180-1B(α) (Figure 9B). A redigestion of the resistant heptasaccharide with α1,2-mannosidase did not release any radioactivity into smaller fragments. (v) The pentasaccharide from (iv) was fully susceptible to cleavage by endo D and gave a product that comigrated on paper with Man(6←1αMan)α-1→3Manβ1→4(3)GlcNAc (Figure 9C). (vi) Affinity chromatography of saccharide VII from the strain X-2180-1B(α) preparation showed that the saccharide was fully bound to Con A–Sepharose and required elution with 0.1 M methyl α-mannoside to release it from the column (Figure 7C). On the other hand, similar chromatography of saccharide VII from the strain ATCC 24297 preparation exhibited 93% binding to the lectin, and 7% of the radioactivity was obtained in the run-through fraction with the elution buffer (Figure 7C). (vii) A methylation analysis of radioactivity eluted from a Con A–Sepharose column with 100 mM methyl α-mannoside gave 2,4-di-*O*-methyl-, 3,4,6-tri-*O*-methyl-, and 2,3,4,6-tetra-*O*-methylmannose as the products (Figure 5G). (viii) Acetolysis of $NaBH_4$-reduced saccharide VII released [^{14}C]mannose and a labeled Man$_4$GlcNAcGlcNAc$_{OH}$ fragment (Figure 9D).

All of the above data indicate that the lipid-linked heptasaccharide synthesized by microsomes from strain X-2180-1B(α) is compatible with the structure Manα1→2Manα1→-2Man(6←1αMan)α1→3Manβ1→4(3)GlcNAcβ1→4(3)-GlcNAc (saccharide VII-1). This is also the predominant heptasaccharide synthesized by microsomal preparation of strain ATCC 24297 and the major lipid-linked heptasaccharide synthesized by animal systems (Chapman et al., 1979; Vijay & Perdew, 1980).

The minor heptasaccharide fraction that failed to bind to the Con A–Sepharose column was resistant to digestion by α1,2-mannosidase. Upon methylation, it gave 2,4-di-*O*-methyl-, 2,4,6-tri-*O*-methyl-, and 2,3,4,6-tetra-*O*-methylmannose as products (Figure 5H). The only structure that is compatible with these data and the structural analysis given above is Manα1→3Manα1→3Man(6←1αMan)α1→-3Manβ1→4(3)GlcNAcβ1→4(3)GlcNAc (saccharide VII-2). Its synthesis may be considered to be due to the incorporation

of a mannose residue in α1,6 linkage to the β mannose of saccharide VI-2.

Discussion

Studies from several laboratories have shown that both in vitro (Parodi, 1978; Lehle, 1980; Trimble et al., 1980) and in vivo (Lehle et al., 1980) preparations from *S. cerevisiae* synthesize lipid-linked oligosaccharides that appear to be intermediates for the biosynthesis of mannoproteins of this organism. The overall composition of the largest lipid-linked oligosaccharide, $Glc_3Man_9(GlcNAc)_2$, synthesized by yeast, is the same as that of the precursor involved for the biosynthesis of asparagine-linked glycoproteins in animal cells. Recently, a major lipid-linked sequence for the assembly of the precursor oligosaccharide for both in vivo (Chapman et al., 1979) and in vitro (Vijay et al., 1980; Vijay & Perdew, 1980; Vijay & Perdew, 1982; Rearick et al., 1981) preparations from animal cells was proposed. Further, it was shown that a number of in vitro synthesized lipid-linked oligosaccharides had minor isomers whose precise physiological significance is still uncertain. Since the asparagine-linked core portion of yeast mannan was shown to have some structural differences from the high mannose oligosaccharides of animal glycoproteins (Nakajima & Ballou, 1975a), it might not be assumed a priori that the sequence of the lipid-linked assembly of the former is the same as that in animal systems. Also, the core section of *S. cerevisiae* mannoproteins is microheterogeneous (Nakajima & Ballou, 1975b). Moreover, the extent of knowledge of glycoprotein processing in the fungi is not as well developed as the same for the biosynthesis of animal glycoproteins.

The biosynthesis of mannosylphosphoryl-, *N*-acetylglucosaminylpyrophosphoryl- and *N,N'*-diacetylchitobiosylpyrophosphoryldolichol by the yeast microsomal systems has been reported earlier (Parodi, 1978; Lehle & Tanner, 1978). Even though the in vitro synthesized lipid-linked oligosaccharides are not uniformly labeled, most likely due to the presence of endogenous glycolipids of different degrees of polymerization, the results of this investigation show that the major lipid-linked oligosaccharides up to heptasaccharide in two different strains of *S. cerevisiae* are identical with the intermediates in animal systems. Thus, at least this portion of the assembly process seems to be common to animal as well as fungal systems. Earlier, it was shown by Lehle (1980) that GDP-mannose appeared to be a donor of the first four (or perhaps five) mannose residues to the lipid-linked *N,N'*-diacetylchitobiose whereas the next four to five mannosyl residues of the precursor required the intermediary synthesis of mannosylphosphoryldolichol. This is also analogous to the lipid-linked assembly process in the animal systems (Spencer & Elbein, 1980; Datema et al., 1980; Banerjee et al., 1981; Rearick et al., 1981; Schutzbach et al., 1980).

The precise reason for the biosynthesis of the minor isomers within the tetra- through heptasaccharides is not entirely clear. The minor isomers synthesized by the yeast microsomal system are structurally different from those synthesized by the in vitro animal systems. However, saccharide IV-2 is identical with the endogenous lipid-linked tetrasaccharide isolated from rabbit liver (Jensen & Schutzbach, 1981); in this system it was shown to be capable of serving as a mannosyl acceptor to give lipid-linked saccharide V-1 as the product. In experiments not reported here, we were unable to detect any α-mannosidase activity in yeast microsomes under conditions that were employed for the biosynthesis of lipid-linked oligosaccharides by using [^{14}C]mannose-labeled $Man_9(GlcNAc)_2$, characterized earlier (Vijay & Perdew, 1980), and a mixture of [^{14}C]mannose-labeled lipid-linked tetra- through undecasaccharides and monitoring for the release of [^{14}C]mannose. One explanation for the biosynthesis of minor isomers within these glycolipids is the lack of absolute specificity of the mannosyltransferases toward the acceptor glycolipids. Any lipid-linked oligosaccharide that is not a physiologically important substrate along the biosynthetic pathway would be inappropriate for the next glycosyltransferase and would tend to accumulate. Indeed, Rearick et al. (1981) indicated that this might be the case in their studies with in vitro assembly of lipid-linked oligosaccharides by a membrane preparation from Thy-1$^-$, class E lymphoma cells. Supplementation of the in vitro biosynthetic system for the bovine mammary tissue with dolichol phosphate markedly reduces the relative amounts of the minor isomeric lipid-linked oligosaccharide in favor of a more ordered mode of elongation (Vijay & Perdew, 1980; Perdew, 1981) similar to that proposed for CHO cells (Chapman et al., 1979). Thus, a limitation of the carrier lipid might be responsible for the biosynthesis of isomeric lipid-linked saccharides in in vitro systems. However, we were unable to stimulate the synthesis of lipid-linked oligosaccharides by the inclusion of detergent-dispersed dolichol phosphate in the yeast biosynthetic system, even though such a supplementation stimulated the biosynthesis of mannosylphosphoryldolichol severalfold. Possibly, the detergents Triton X-100 and NP-40, employed by us, might be inhibitory to other glycosyltransferases in yeast. Until the enzymology of lipid-linked assembly is defined in both animal tissues and the fungi, it is not possible to draw any definitive conclusions regarding origin and function of the minor isomeric lipid-linked oligosaccharides.

Parodi (1981) extended his studies with the biosynthesis of mannoproteins in *S. cerevisiae* to include both mnn 1 and mnn 2 mutants of the wild-type X-2180. The mnn 1 mutant lacks an α1,3-mannosyltransferase that transfers a terminal α1,3-linked mannose to both O-linked side chains, the tetrasaccharide side chain in the core portion, and the outer region of the N-linked polysaccharide (Ballou, 1976). The mnn 2 mutant makes a mannan that lacks all side chains in the outer region; this mutation causes an alteration (or deletion) of the α1,2-mannosyltransferase that attaches the first mannose residue to the α1,6-linked backbone of the N-linked polysaccharide (Ballou, 1976). The lipid-linked precursor for the wild type as well as the mnn 1 and mnn 2 mutants had the same overall structure, i.e., $Glc_3Man_9(GlcNAc)_2$. Only after transfer to the protein acceptor did one mannosyl residue appear to be trimmed off along with the three glucosyl residues before (or concomitant with) the elongation reactions for the construction of the outer region. However, a comparison of the inner core of the *S. cerevisiae* mannan with that of the high mannose glycoproteins of animal origin indicates at least three terminal mannose residues in the side chains that are α1,3 linked instead of being in the usual α1,2 attachment. It could be that the biosynthesis of the lipid-linked octa- through undecasaccharides might not be the same as in animal systems, thereby resulting in the synthesis of lipid-linked oligosaccharides that are structurally different from the same in the major assembly sequence in animal systems. Or, there might be more than one mannose residue that is trimmed off after the transfer of the precursor oligosaccharide to the mannan acceptor. Such a processing may then be followed by the addition of α1,3-linked mannoses. Alternatively, even though the excellent classical approach for structural analysis, coupled with immunological evidence for terminally α1,3-linked mannose residues within the side chains of the inner core of *S. cerevisiae* mannan, were employed by Nakajima

and Ballou (1974), several assumptions were used to interpret the structural data. A minor revision of this structure could then explain the biosynthetic and the processing sequences once the latter are completed.

Our primary objective for undertaking this study was to examine the details of the lipid-linked assembly in yeast since mannoproteins of *S. cerevisiae* appear to be prototypes of the asparagine-linked glycoproteins during the evolutionary development of eukaryotes. While the lipid-linked biosynthesis of the inner core of the mannan is analogous to the synthesis of high mannose glycoproteins and the trimannosylchitobiosyl portion of the complex glycoproteins in animals, the direct transfer of mannose residues from GDP-mannose to the outer region of the polysaccharide is somewhat similar to the direct incorporation of terminal sugars from corresponding sugar nucleotides for the biosynthesis of complex glycoproteins (Schachter & Roseman, 1980). Further, the transfer of the phosphate group into the outer region as mannose 1-phosphate and its presence as a "capped" diester are reminiscent of an early evolutionary mechanism for the generation of the recognition marker required for targeting glycoproteins into lysosomes. The transfer of *N*-acetylglucosamine 1-phosphate to the protein-linked high mannose oligosaccharide (Hasilik et al., 1981; Reitman & Kornfeld, 1981; Waheed et al., 1981a) and the excision of GlcNAc (Varki & Kornfeld, 1981; Waheed et al., 1981b) have been shown to be important reactions for the biosynthesis and intracellular localization of lysosomal glycoproteins in animal cells.

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