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Biochemistry

Carbohydrate chains on yeast carboxypeptidase Y are phosphorylated

(glycoprotein high mannose oligosaccharides/nuclear magnetic resonance/α-mannosidase/phosphodiester)

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ABSTRACT **Carboxypeptidase Y, a vacuolar enzyme from *Saccharomyces cerevisiae*, was digested with *endo*-β-*N*-acetyl-D-glucosaminidase H to release the four oligosaccharide chains that are linked to asparagine in the glycoprotein. The oligosaccharides were fractionated into a neutral and acidic component, and the latter proved to phosphorylated. From its gel filtration pattern, the neutral fraction was shown to be a mixture of at least four homologs, the smallest of which had a proton NMR spectrum almost identical to that given by an IgM oligosaccharide with eight mannoses and one *N*-acetylglucosamine [Cohen, R. E. & Ballou, C. E. (1980) *Biochemistry* 19, 4345–4358]. The yeast oligosaccharide has one additional mannose unit in an α1→3 or α1→6 linkage, whereas the larger homologs appear to have two, three, and four more mannose units. One phosphorylated oligosaccharide with a mannose/phosphate ratio of 12.5 was reduced with NaB^3H_4 and then subjected to mild acid hydrolysis. This released mannose and mannobiose that were glycosidically linked to the phosphate group, whereas complete acid hydrolysis yielded D-mannose 6-phosphate. The recovered oligosaccharide phosphomonoester, which contained 11 or 12 mannose units, was digested exhaustively with α-mannosidase, and the product of this reaction was treated with alkaline phosphatase, which yielded radioactive $Man_3GlcNAcH_2$. These results suggest that the mannosidase-resistant phosphorylated oligosaccharide has the structure Man→P→6αMan→αMan→6βMan→4GlcNAcH_2, in which some of the phosphate groups are substituted with mannobiose instead of mannose. A second phosphorylated oligosaccharide with a mannose/phosphate ratio of 6.5 probably contains two phosphodiester groups, but its structure has not been investigated in detail.**

The yeast *Saccharomyces cerevisiae* has extracellular periplasmic glycoproteins (mannoproteins), such as invertase, that contain ≈50% mannose (1) and intracellular glycoproteins, such as carboxypeptidase Y, that contain 15% mannose (2) and are similar in some respects to mammalian glycoproteins (3). The asparagine-linked carbohydrate chains of the external invertase contain 100–150 mannose units that are differentiated into a core unit of ≈11 mannoses and an outer chain of 90–140 mannoses (4). From the composition and number of the carbohydrate chains in carboxypeptidase Y, the average chain size appears to be ≈13 mannoses (5)—i.e., the size of a core unit. From these facts, one might postulate that the externalization of a mannoprotein is associated with or dependent on the addition of the outer chain and that intracellular mannoproteins lack a signal that specifies outer-chain addition (6). Although it is an attractive idea, a strong argument against this hypothesis is our recent isolation of *S. cerevisiae* mutants that make and secrete mannoproteins, including invertase, that appear to possess only the core oligosaccharide units (7).

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To determine whether the carbohydrate chains have any role in specifying the compartmentation of glycoproteins in yeast, we have undertaken a detailed comparison of the core oligosaccharide units from intracellular and extracellular mannoproteins. This preliminary report on the carbohydrate chains from carboxypeptidase Y shows that they are similar to oligosaccharides from mammalian glycoproteins and that some of the chains are phosphorylated. Because carboxypeptidase Y is found in the yeast vacuole (8), an organelle somewhat analogous to the lysosome of higher organisms, the phosphorylation of the carbohydrate chains may have a role in determining the localization of the enzyme, as it appears to have with some lysosomal enzymes (9).

MATERIALS AND METHODS

Materials. Ion exchange resins and gel filtration materials came from Bio-Rad and phenylmethanesulfonyl fluoride (toluenesulfonyl fluoride) was from Sigma. Bacterial alkaline phosphatase was from Worthington, jack bean α-mannosidase was prepared according to Li (10), and *endo*-β-*N*-acetyl-D-glucosaminidase H was purified from *Streptomyces plicatus* (American Type Culture Collection 27800) according to Tarentino *et al.* (11). Carboxypeptidase Y was isolated from compressed bakers' yeast (Universal Foods, San Francisco) by the procedure of Kuhn *et al.* (12). Reference oligosaccharides were from an earlier study (13).

Methods. Alkaline phosphatase digestion was done for 24 hr in 1.0 M Tris·HCl (pH 8.0); α-mannosidase digestion was for 24 hr in 50 mM NaOAc, pH 4.5/0.1 mM $ZnCl_2$; and endoglucosaminidase H digestion was for 96 hr in 50 mM Na citrate (pH 5.5). All reactions were carried out at 37°C. Carboxypeptidase Y was treated with toluenesulfonyl fluoride according to Kuhn *et al.* (14). Mannose 6-phosphate was determined by a coupled enzyme assay (15) as modified by Hashimoto *et al.* (16). A correction was made for the amount of NADPH produced by an acid hydrolysate of bovine serum albumin. Partial acetolysis of oligosaccharides was carried out in acetic acid/acetic anhydride/sulfuric acid, 10:10:1 (vol/vol) at 40°C for 7 hr (17), and the recovered acetylated products were deacetylated in methanol containing Na methoxide. Mild acid hydrolysis to cleave glycosylphosphate bonds was done in 0.01 M HCl at 100°C for 30 min. Carboxypeptidase Y was methylated by a standard procedure (13).

Oligosaccharide samples (50–500 μg) were reduced in 500 μl of 0.1 M $NaHCO_3$ by adding 200 μCi (1 Ci = 3.7×10^{10} becquerels) of NaB^3H_4 as a dimethyl sulfoxide solution (4 mCi/ml). After 12 hr at 23°C, 5 mg of $NaBH_4$ was added and the reaction was allowed to proceed for another 12 hr. Dowex 50 (H^+) was added until effervescence ceased, the tube contents were filtered through a Pasteur pipette plugged with glass wool, and the filtrate was evaporated to dryness under reduced pressure. One drop of glacial acetic acid and 1 ml of methanol were

added to the residue, and the mixture was refluxed for 1 min and then evaporated to dryness under a stream of N_2. This treatment was repeated four times. For neutral samples, an acidic noncarbohydrate radioactive contaminant was removed by passing a water solution of the reduced product through a small column of Dowex 1 (acetate). Acidic reduced oligosaccharides were purified on a Bio-Gel P-4 (>400 mesh) column (1 × 25 cm) in 0.1 M acetic acid. Finally, the reduced compounds were desalted by passage through a Sephadex G-10 column (0.5 × 25 cm) in water.

Oligosaccharide sizes were estimated by gel filtration on a calibrated Bio-Gel P-4 column (>400 mesh) (0.6 × 115 cm) in 0.1 M NH_4OAc containing 0.02% NaN_3 to prevent microbial growth. Fractions of 3 drops were collected, and the elution positions were determined relative to the reference compounds βMan→4GlcNAcH_2, αMan→3βMan→4GlcNAcH_2, αMan→2αMan→2αMan→3βMan→4GlcNAcH_2, Man_6GlcNAcH_2 (from ovalbumin) (13), Man_8GlcNAcH_2 (from IgM) (13), and Man_{11}GlcNAcH_2 (from yeast mannoprotein) (7). On this same column, bovine serum albumin was eluted at fraction 60 and mannose was eluted at fraction 176.

Polyacrylamide gel (10%)/0.1% $NaDodSO_4$ electrophoresis was done by the discontinuous system of Laemmli (18), with a running buffer of Tris·HCl (pH 7.2). Samples were reduced with 1% mercaptoethanol/1% $NaDodSO_4$ at 100°C for 3 min. Gels were stained for protein with Coomassie brilliant blue R-250.

^1H NMR and ^{31}P NMR were carried out as described (13, 16).

RESULTS

Isolation of Carboxypeptidase Y and Digestion with Endoglucosaminidase H. From 100 lb of yeast cake, ≈500 mg of pure carboxypeptidase Y was obtained. As noted by Kuhn *et al.* (12), two peaks of enzyme activity were found at the DEAE-Sephadex A-50 isolation step, here designated CPY-I and CPY-II. These two peaks were separated, and each gave a single peak when it was rechromatographed. The carbohydrate content of CPY-I was 14.4% and that of CPY-II was 14.7%. Hasilik and Tanner (5) have reported 14.1% carbohydrate for the enzyme. Both carboxypeptidase preparations contained organic phosphate, the mannose/phosphate molar ratios being 12.5 for CPY-I and 10.8 for CPY-II. Both fractions of carboxypeptidase Y were inactivated with toluenesulfonyl fluoride (14) to prevent autodigestion and digestion of the endoglucosaminidase H during treatment to release the carbohydrate chains. The yield of inactivated CPY-I was 260 mg and that of inactivated CPY-II was 230 mg. Both preparations gave single major bands that were indistinguishable on acrylamide/$NaDodSO_4$ gel electrophoresis (Fig. 1).

Methylation of intact CPY-I, followed by hydrolysis, reduction, and acetylation of the methylated derivative, yielded the alditol acetates of 2,3,4,6-tetra-*O*-methylmannose (5 mol), 2,4,6-tri-*O*-methylmannose (3 mol), 3,4,6-tri-*O*-methylmannose (2 mol), 2,4-di-*O*-methylmannose (1 mol), and 3,4,-di-*O*-methylmannose (2 mol). The two di-*O*-methyl derivatives are those expected for the branching at positions 2 and 3 of the 1→6-linked backbone mannose that is characteristic of yeast glycoprotein core oligosaccharides (13). Also typical are the two tri-*O*-methyl ethers that reflect the presence of mannose in 1→2 and 1→3 linkage.

Samples of inactivated CPY-I and CPY-II (150 mg each) were digested with 0.75 unit of endoglucosaminidase H for 96 hr (19). As shown in Fig. 1, both carboxypeptidase preparations were converted quantitatively to single faster-migrating protein bands that were again indistinguishable. The enzyme digests were then fractionated on a Bio-Gel P-30 (100–200 mesh) column (2 × 90 cm) by elution with water (Fig. 2). The separated protein peak (fractions 23–31) contained very little mannose; 95% of the mannose was distributed in a neutral peak (fractions 78–93) and a broader peak (fractions 32–71) associated with most of the phosphate. The latter was divided into three fractions: fraction a with a mannose/phosphate ratio of 6.5, fraction b with

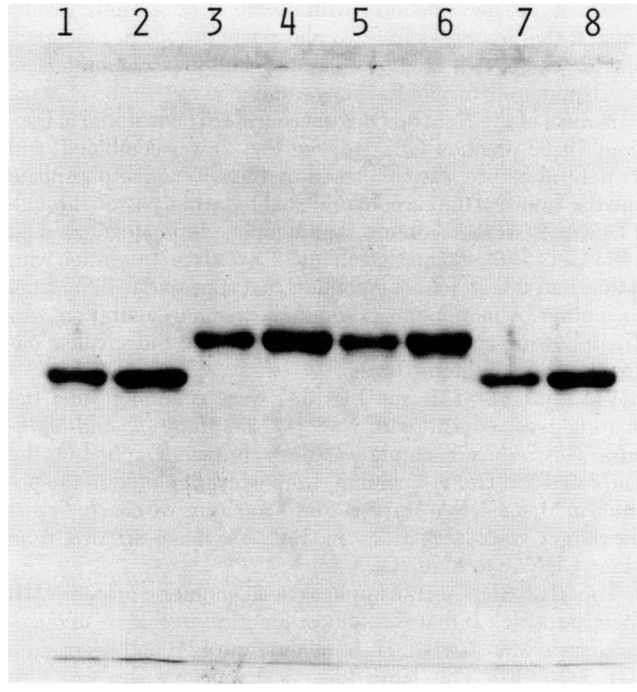

FIG. 1. Polyacrylamide/$NaDodSO_4$ gel electrophoresis of carboxypeptidase preparations. Lanes 3 and 4, undigested inactivated CPY-I at 2 and 4 μg of protein per lane, respectively; lanes 5 and 6, undigested inactivated CPY-II at 2 and 4 μg; lanes 1 and 2, endoglucosaminidase H-digested CPY-I at 2 and 4 μg of protein per lane; lanes 7 and 8, endoglucosaminidase H-digested CPY-II at 2 and 4 μg of protein per lane.

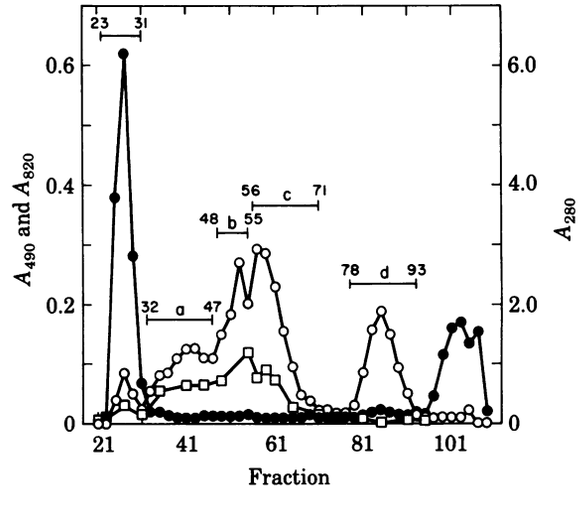

FIG. 2. Fractionation of endoglucosaminidase-digested inactivated CPY-II on a Bio-Gel P-30 column. The elution by water of carbohydrate (A_{490}, ○), phosphate (A_{820}, □), and protein (A_{280}, ●) are shown. Only 5% of the carbohydrate was eluted with the protein peak (fractions 23–31) corresponding to the carboxypeptidase. Phosphate-free carbohydrate (fractions 78–93) was 20% of the total and phosphate-containing carbohydrate (fractions 32–71) was 71%. The latter was divided into three parts, CPY-IIa (Man/P = 6.5), CPY-IIb (Man/P = 8.0), and CPY-IIc (Man/P = 12.5).

a ratio of 8.0, and fraction c with a ratio of 12.5. These results suggest that fraction a has two phosphate groups per oligosaccharide chain, that fraction c has one, and that fraction b is an overlapping mixture of fractions a and c.

Nature of the Neutral Oligosaccharide. The material in fractions 78–93 (fraction CPY-IId; see Fig. 2) was combined, and its neutral nature was confirmed by the absence of phosphate and the failure of the carbohydrate to bind when passed through a Dowex 1 (acetate) column. The sample, chromatographed on a Bio-Gel P-4 (>400 mesh) column (2 × 190 cm) by elution with water, had at least four components that appeared to differ from each other by increments of about one mannose unit (Fig. 3*A*). The following characterization was limited to the smallest oligosaccharide of the mixture.

Fractions 114–121 (see Fig. 3*A*) were combined, and the material was reduced with NaB^3H_4 (see *Materials and Methods*). This reduced sample was then chromatographed on the calibrated Bio-Gel P-4 column, from which it eluted in the position of $Man_9GlcNAcH_2$ (Fig. 3*B*). From this, we conclude that the larger oligosaccharides in Fig. 3*A* range in size from $Man_{11}GlcNAc$ to $Man_{13}GlcNAc$.

The $Man_9GlcNAcH_2$ sample gave an anomeric proton NMR spectrum (Fig. 4) that was almost identical to that of an oligosaccharide $Man_8GlcNAcH_2$ obtained from IgM and designated rGP-563-I (13). This latter oligosaccharide has the structure shown in bold type in Fig. 4. The carboxypeptidase oligosaccharide has one more mannose unit, however, and, from the shoulder (*g*) on peak *b*, we conclude that about half of the chains have an unsubstituted α1→6-linked mannose, whereas the excess area under peak *a,e,e'* would be consistent with about half of the chains having an additional terminal α1→3-linked mannose. The postulated modifications are shown in italics on the structure in Fig. 4.

Nature of the Phosphorylated Component of Carboxypeptidase Y. CPY-I and CPY-II were hydrolyzed in 1 M trifluoroacetic acid at 120°C for 2 hr. The acid was evaporated under a stream of N_2, and the residue was assayed enzymically for D-mannose 6-phosphate (15, 16). The results (Table 1) show that the phosphate was almost quantitatively accounted for as mannose 6-phosphate.

Phosphorylated oligosaccharide CPY-IIc [fractions 56–71 (see Fig. 2)] was passed through a Bio-Gel P-4 (>400 mesh) column (2 × 190 cm) and eluted with 0.1 M NH_4OAc in 3-ml

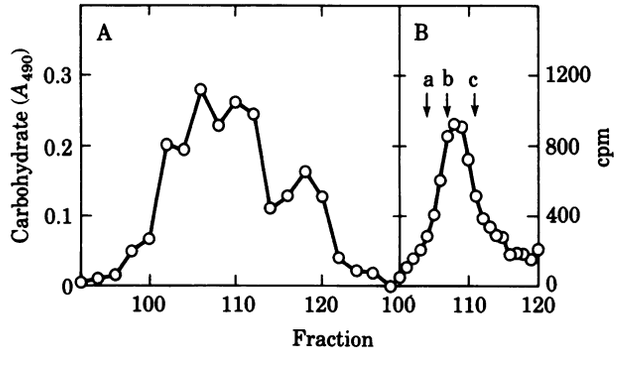

FIG. 3. Fractionation of CPY-IId neutral oligosaccharide component from Fig. 2. (*A*) Separation on a Bio-Gel P-4 (>400 mesh) column (2 × 190 cm) by elution with water. Carbohydrate (A_{490}) is indicated. High-performance liquid chromatography (not shown) gave somewhat better separation, but the result confirmed this general distribution of fragments. (*B*) Elution of the NaB^3H_4-reduced smallest component (fractions 114–121) on the calibrated Bio-Gel P-4 (>400 mesh) column (0.6 × 115 cm) by 0.1 M NH_4OAc. Elution positions of $Man_{10}GlcNAcH_2$ (a), $Man_9GlcNAcH_2$ (b), and $Man_8GlcNAcH_2$ (c) are indicated.

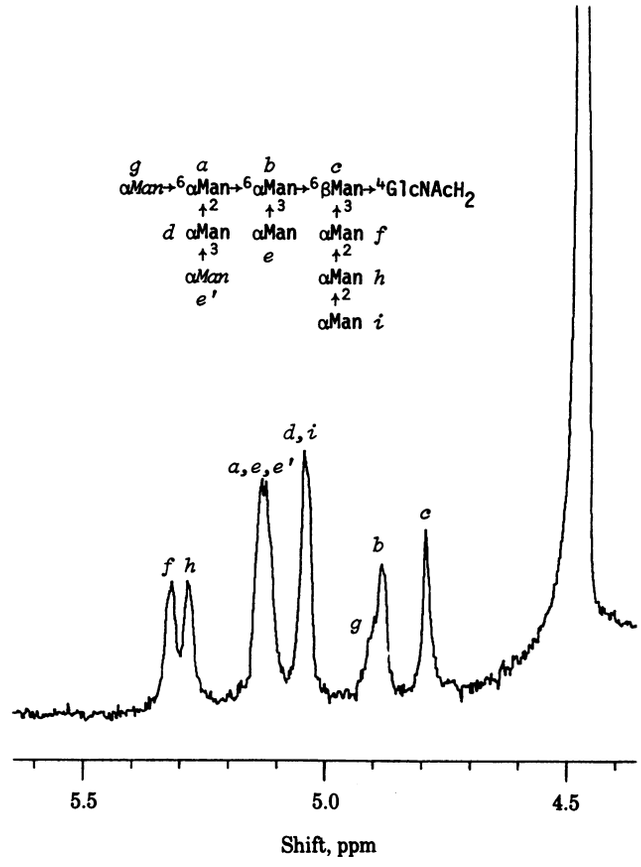

FIG. 4. Anomeric proton NMR spectrum of reduced small oligosaccharide ($Man_9GlcNAcH_2$) from Fig. 3*B*. Italicized letters correlate the different sugar units with the resonance lines in the spectrum. Integrations of the signals were as follows: *a,e,e'* = 2.38; *b,g* = 1.36; *c* = 1.0; *d,i* = 2.04; *f* = 0.95; *h* = 0.99. The $Man_8GlcNAcH_2$ structure shown in bold type is identical with an IgM oligosaccharide (13), and the CPY-IId $Man_9GlcNAcH_2$ appears to be a 1/1 mixture of isomers, each with one of the italicized mannose units in α1→3 (*e'*) or α1→6 (*g*) linkage. The large signal at δ4.5 is H^2HO.

fractions. The majority of the carbohydrate appeared in a symmetrical peak between fractions 97 and 113. The combined material was analyzed by proton-decoupled ^{31}P NMR, and it gave a single signal at −1.7 ppm (pH 7.0 in 0.1 M Na EDTA), a chemical shift characteristic of phosphate diesters (20).

S. cerevisiae mannoproteins often contain diesterified phosphate in which one component of the diester is mannose or mannobiose in glycosyl linkage that is subject to release by mild acid hydrolysis (21). Phosphorylated CPY-IIc oligosaccharide was heated at 100°C for 30 min in 0.01 M HCl, and the products were separated on a Bio-Gel P-2 (>400 mesh) column (0.2 × 114 cm) by elution with 0.1 M NH_4OAc (Fig. 5). Peaks corre-

Table 1. D-Mannose 6-phosphate from hydrolyzed carboxypeptidase Y

Sample	Organic phosphate added, nmol	NADPH produced, nmol	NADPH per phosphate*	
			Found	Corrected
CPY-I	6.0	7.8	1.3	0.9
CPY-II	6.0	9.0	1.5	1.1

* The high values given by carboxypeptidase Y appear due to contamination of the assay enzymes by an amino acid oxidase; the corrected values were obtained by subtracting the amount of NADPH produced with an equivalent amount of hydrolyzed bovine serum albumin.

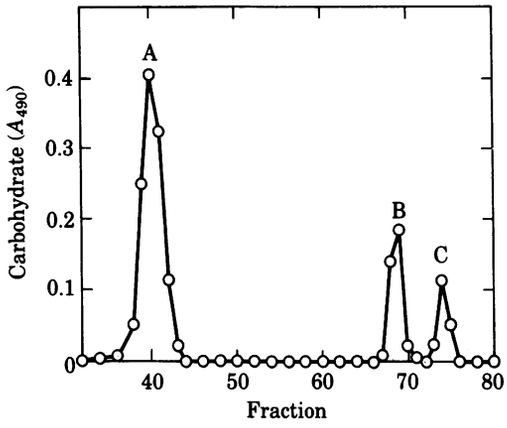

FIG. 5. Fractionation of the mild acid hydrolysis products from phosphorylated CPY-IIc oligosaccharide. The hydrolyzed sample was separated on a Bio-Gel P-2 column, and the fractions were analyzed for carbohydrate (A_{490}). For generation of peaks B and C, which correspond to mannobiose and mannose, respectively, the samples taken were 10-fold larger than for peak A, which is the oligosaccharide phosphomonoester residue. The molar ratio B/C is 0.82, and the weight ratio A/(B + C) is 21. The material in peak A was subjected to a second mild acid hydrolysis, which released an additional amount of mannobiose such that the combined ratio A/(B + C) for the two hydrolyses became 10.6.

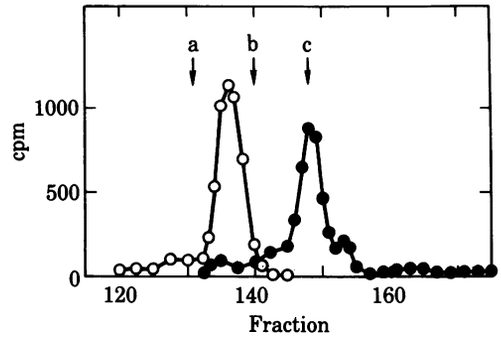

FIG. 6. Location of mannose phosphate in the acid-stable oligosaccharide phosphate. The NaB^3H_4-reduced oligosaccharide CPY-IIc was hydrolyzed in 0.01 M HCl at 100°C for 30 min and digested with α-mannosidase; the recovered acidic product was then treated with alkaline phosphatase. The elution position of the neutral radioactive product (○) corresponds to that of $Man_3GlcNAcH_2$. This product was recovered and subjected to partial acetolysis at 40°C for 7 hr, and the deacetylated products were run on the same column (●). The major radioactive peak corresponds to $ManGlcNAcH_2$. The elution positions of $Man_4GlcNAcH_2$ (a), $Man_2GlcNAcH_2$ (b), and $Man_1GlcNAcH_2$ (c) are indicated by arrows.

sponding to mannose and mannobiose were obtained, and their identities were confirmed by paper chromatography (not shown). The weight ratio oligosaccharide residue/mannose plus mannobiose was 21, whereas a ratio of about 12 would be expected if all of the phosphate groups were esterified with mannose. Incomplete hydrolysis of the diester bond is the most likely explanation for this result because subsequent phosphatase digestion gave only a 70% yield of neutral oligosaccharide. In confirmation of this conclusion, a second mild acid hydrolysis of the recovered oligosaccharide gave sufficient additional mannose to bring the weight ratio oligosaccharide/mannose plus mannobiose to 10.6.

A sample of the acidic CPY-IIc oligosaccharide was reduced with NaB^3H_4, and the product was subjected to mild acid hydrolysis and treatment with alkaline phosphatase to dephosphorylate the monoester oligosaccharide component. This gave a neutral radioactive oligosaccharide that was separated into two peaks on the calibrated Bio-Gel P-4 column. These two appear to differ by one mannose unit, and their sizes correspond to $Man_{11}GlcNAcH_2$ and $Man_{12}GlcNAcH_2$ (not shown).

Location of the Mannose 6-Phosphate in the Acid-Stable Oligosaccharide. Strong acid hydrolysis of intact carboxypeptidase Y gives mannose 6-phosphate (see above), so this structure must be present in the acidic oligosaccharide recovered after mild acid hydrolysis. The location of the mannose phosphate was determined by subjecting the NaB^3H_4-reduced oligosaccharide to exhaustive digestion with α-mannosidase. The recovered acidic radioactive product was dephosphorylated with alkaline phosphatase and then fractionated on the calibrated Bio-Gel P-4 column (Fig. 6). It eluted in the position of $Man_3GlcNAcH_2$. Assuming the radioactive product was derived from an oligosaccharide such as that in Fig. 4, this suggested three possible structures: αMan→6αMan→6βMan→4GlcNAcH$_2$, αMan→2(or 3)αMan→6βMan→4GlcNAcH$_2$, and αMan→2αMan→3βMan→4GlcNAcH$_2$. To discriminate between these, we used partial acetolysis (17); only the oligosaccharides with 1→6 linkages would be degraded readily in this reaction. As shown in Fig. 6, the acetolysis of $Man_3GlcNAcH_2$ labeled only in the glucosaminitol residue yields a radioactive product having the elution position of $ManGlcNAcH_2$. This is consistent with either of the first two structures above. Because the original phosphorylated oligosaccharide (CPY-IIc) contains 11 or 12 mannoses, however, the backbone probably has four 1→6-linked mannose units, which would preclude attachment of phosphate to position 6 of the third mannose in the mannosidase-resistant tetrasaccharide. From this consideration, we favor the structure αMan→2 (or 3) αMan→6βMan→4GlcNAcH$_2$ for this oligosaccharide.

DISCUSSION

Yeast carboxypeptidase Y contains phosphate in diester form that is linked as mannosylphosphate and mannobiosylphosphate units to position 6 of mannose in some of the oligosaccharide units of the glycoprotein. These observations show a striking parallel between this yeast vacuolar hydrolytic enzyme and the lysosomal enzymes of higher organisms, some of which are also phosphorylated (9). Whether the phosphorylated oligosaccharide chains of carboxypeptidase Y specify the localization of this enzyme in the vacuole is unknown, but it is certain that the mere presence of phosphate in a mannoprotein does not determine location; some secreted yeast mannoproteins also carry mannosylphosphate and mannobiosylphosphate groups in the outer chain (22). The novelty in the present report is the discovery of phosphate in the core oligosaccharide of an intracellular yeast mannoprotein.

It has been reported (12), and we confirm, that partially purified carboxypeptidase Y is resolved on DEAE-Sephadex into two peaks of enzyme activity. It is possible that this separation is based on different phosphate contents. The molar ratios of mannose/phosphate were 12.5 in CPY-I and 10.8 in CPY-II. As carboxypeptidase contains ≈50 mannose units per molecule (5), this means that there may be four phosphates in CPY-I and five in CPY-II. The additional phosphate group in CPY-II would be consistent with its later elution from DEAE-Sephadex. In our isolation, the ratio CPY-I/CPY-II was ≈0.8, whereas Kuhn *et al.* (12) found a ratio of about 0.5.

The finding of four or five phosphate groups per enzyme molecule and the fact that one-fourth of the four oligosaccharide chains are not phosphorylated suggests that some oligosaccharide chains must carry more than one phosphate group. The elution pattern of the oligosaccharides from the Bio-Gel P-30

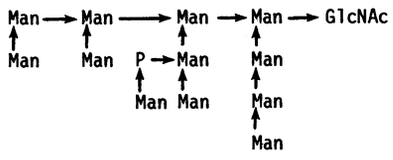

FIG. 7. Proposed partial structure for oligosaccharide CPY-IIc. Other homologs in the mixture contain mannobiose in place of mannose on the phosphate group and an additional mannose unit elsewhere in the core oligosaccharide.

column does reveal heterogeneity in the extent of phosphorylation, and the early fraction (CPY-IIa) appears to have about twice as much phosphate as the later one (CPY-IIc).

By ^{31}P NMR we have shown that all of the phosphate in carboxypeptidase Y is diesterified and, because strong acid hydrolysis yields mannose 6-phosphate quantitatively, it is clear that the phosphate is esterified to position 6 of mannose units in some of the oligosaccharide chains. In most or all of the phosphodiester units, the other attached group is mannose or mannobiose in an acid-labile glycosylphosphate linkage. The phosphorylated mannose unit of the carboxypeptidase Y oligosaccharide CPY-IIc is probably attached as a side chain to the first $\alpha 1 \rightarrow 6$-linked mannose unit of the backbone by an $\alpha 1 \rightarrow 2$ or $\alpha 1 \rightarrow 3$ linkage (Fig. 7). If the phosphorylated oligosaccharides are related structurally to those of the outer chain (22), the linkage is probably $\alpha 1 \rightarrow 2$. This structure has obvious similarity to that reported by Tabas and Kornfeld (23) for a mouse lymphoma β-glucuronidase in which *N*-acetylglucosamine phosphate is esterified to the core oligosaccharide.

All of the carbohydrate chains on carboxypeptidase Y are not phosphorylated, and one of the neutral oligosaccharides was shown to have a structure almost identical to that of an IgM chain with eight mannoses (13). The unphosphorylated carboxypeptidase Y oligosaccharide fraction (CPY-IId) is composed of at least four homologs that appear to have 9, 11, 12, and 13 mannoses, respectively. This neutral fraction makes up about one-fourth of the total oligosaccharide component and could all be localized at a particular site in the polypeptide chain, but we have not investigated this feature. Methylation of intact carboxypeptidase Y showed the presence of 2- and 3-*O*-substituted, as well as 2,6- and 3,6- doubly *O*-substituted mannoses. Because the carbohydrate chains are heterogeneous, the observed ratios of these units do not fit any single structure well, but all of the linkages are expected in the yeast core oligosaccharides. The somewhat high proportion of $1 \rightarrow 3$-linked mannose is consistent with the presence of this linkage in the mannobiose unit previously found attached to phosphate in the mannoprotein outer chain (22).

That the observed heterogeneity of the carboxypeptidase Y oligosaccharides results from phosphatase and mannosidase digestion during isolation of the enzyme seems unlikely for several reasons. Some of the neutral oligosaccharides are larger than the corresponding part of the acidic ones; none of the oligosaccharides contains monoesterified phosphate, which would presumably be an intermediate in any degradative pathway; and the smallest neutral oligosaccharide detected contained nine mannose units, whereas smaller oligosaccharides are readily formed by digestion with jack bean α-mannosidase.

Yeast mannoproteins show a high species polymorphism (24), and the carbohydrate chains of the external mannoprotein, invertase, are known to be heterogeneous in size (25) and in the structure of the core oligosaccharides (4). As we show in the present study, carboxypeptidase Y, an intracellular mannoprotein that possesses only core oligosaccharides, has a very heterogeneous assortment of carbohydrate chains that differ in both size and state of phosphorylation. An important aim in extending this study will be to elucidate the origin and function of this diversity.

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