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Dolichyldiphosphoryloligosaccharide—protein oligosaccharyltransferase: Solubilization, purification, and properties

(glycoprotein synthesis/glycosylaminyltransferase/soluble membrane protein)

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ABSTRACT **Dolichyldiphosphoryloligosaccharide—protein oligosaccharyltransferase was solubilized from hen oviduct rough endoplasmic reticulum by extraction with 0.2% Nonidet P40. Oligosaccharyltransferase activity was assayed in an incubation mixture containing Glc_n-Man_x-$GlcNAc_2$-diphosphoryldolichol as an oligosaccharyl donor and the ^{125}I-labeled tryptic peptide consisting of residues 29–58 from bovine α-lactalbumin as acceptor. The transferase was purified approximately 2000-fold by fractionation on a bovine α-lactalbumin-Sepharose column; the active material bound quantitatively to the gel and was eluted by removal of divalent cation from the wash buffer. The product of the transferase activity, ^{125}I-glycopeptide, was determined as concanavalin A-agarose-adsorbed radioactivity by a filter disc assay method. ^{125}I-Labeled concanavalin A-agarose-bound product was characterized as a glycopeptide as follows: (*i*) gel filtration behavior on Sephadex G-50; (*ii*) elution from concanavalin A-agarose with 1% α-methyl mannoside; (*iii*) absence of affinity for ricin-Sepharose and loss of affinity for concanavalin A-agarose after treatment with endo-β-*N*-acetylglucosaminidase *H*; (*iv*) enzymatic synthesis of identical product upon using [^3H]oligosaccharyldiphosphoryldolichol and unlabeled peptide acceptor; and (*v*) digestion of ^3H-labeled peptide with Pronase, resulting in the formation of lower molecular weight glycopeptide. Oligosaccharyltransferase activity exhibited an absolute requirement for divalent cations (3 mM Mn^{2+}; Mg^{2+} was 30% as effective), complete dependence on exogenously supplied peptide acceptor (1.33 μg/ml) and oligosaccharyldiphosphoryldolichol (approximately 10 nmol/ml), and an optimum pH between 7 and 7.5.**

It is now well established that the core portion of the glycosylamine-linked carbohydrate sidechains of glycoproteins are biosynthesized by microsomal membrane-associated enzyme systems as complex oligosaccharyldiphosphoryldolichol derivatives and subsequently transferred *en bloc* to specific asparagine residues of nascent polypeptide chains (1). Selected enzymes of the dolichol pathway have been studied in soluble form after extraction of microsomal membranes from a variety of tissues with detergents (2, 3). However, the enzyme involved in the final step in this sequence of reactions, the transferase that catalyzes attachment of the oligosaccharide to an asparaginyl residue of protein to form a glycosylamine linkage has been studied only in intact membrane preparations. A major difficulty in studying this enzyme has been the lack of a convenient, inexpensive, and sensitive assay for oligosaccharyltransferase activity. Thus, generally the activity has been demonstrated by determining the amount of radioactive glycopeptide formed in mixtures containing ^3H- or ^{14}C-labeled oligosaccharyldiphosphoryldolichol and unlabeled proteins (or peptides) as substrates (4, 5). Because it is necessary to prepare these radioactive substrates *in vitro*, their specific radioactivities are low and, consequently, the assay system is relatively insensitive, expensive, and time-consuming. We have now developed a modified assay procedure that overcomes most of these difficulties and provides a basis for more extensive studies on the oligosaccharyltransferase. This enzyme, dolichyldiphosphoryloligosaccharide—protein oligosaccharyltransferase has now been extracted in soluble form from hen oviduct microsomal membranes and purified extensively by affinity chromatography, and the reaction catalyzed by the purified enzyme has been partially characterized.

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EXPERIMENTAL PROCEDURES

Materials. Bovine α-lactalbumin (grade II) was purchased from Sigma, and bovine serum albumin was from Miles. Concanavalin A (Con A)-agarose was obtained from either Sigma or E. Y. Laboratories (San Mateo, CA). Ricin-Sepharose 4B was prepared by the procedure of March *et al.* (6), whereas α-lactalbumin-Sepharose 4B was made according to the procedure of Trayer and Hill (7). GDP-mannose and UDP-*N*-acetylglucosamine were purchased from Sigma, and UDP-[1-^3H]glucose (3.26 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was from New England Nuclear. Sodium [^{125}I]iodide (13–17 mCi/μg) was obtained from Amersham. Pronase (Grade B) was a product of Calbiochem, and endo-β-*N*-acetylglucosaminidase *H* was purchased from Miles. Nuflow cellulose acetate membrane filters (pore size, 0.45 μm; diameter, 25 mm) were manufactured by Courtaulds for Oxoid, Basingstoke, England. All other reagents used were purchased from commercial sources and were of the highest purity available.

Preparation of ^{125}I-Labeled Peptide Acceptor. The tryptic peptide consisting of residues Thr-29 through Lys-58 was prepared from aminoethylated bovine α-lactalbumin by following the procedures of Struck *et al.* (5) and Hart *et al.* (8). The tryptic peptide was labeled with ^{125}I by using chloramine-T (9). The specific activity of the isolated peptide was 2×10^7 cpm/μg, and 100 ng of the radiolabeled peptide was used in a standard assay mixture.

Preparation of Hen Oviduct Microsomal Membranes. Mature laying hens (generally 28 to 33 weeks old), obtained from a local farm, were killed by decapitation and the magnum portion of the oviduct was excised. The tissues were chilled in

Abbreviations: Con A, concanavalin A; NP40, Nonidet P40; Ose, glycose; oligo(Ose)-*PP*-Dol, dolichyldiphosphoryloligosaccharide; αMeMan, α-methyl mannoside. Buffer compositions are: TKMD, 50 mM Tris·HCl, pH 7.7/25 mM KCl/2.5 mM $MgCl_2$/3 mM dithiothreitol; Tris/NP40, 50 mM Tris·HCl, pH 7.5/25 mM NaCl/10 mM $MgCl_2$/20 mM $MnCl_2$/140 mM sucrose/0.2% NP40; TSMS/NP40, 50 mM Tris·HCl, pH 7.5/25 mM NaCl/20 mM $MnCl_2$/140 mM sucrose/0.2% NP40; TSMC, 20 mM Tris·HCl, pH 7.5/0.2 M NaSCN/1 mM $MnCl_2$/1 mM $CaCl_2$.

ice immediately upon removal from the animals. All subsequent procedures were carried out at 0–4°C. The major blood vessels and connective tissue were removed and the magnum was minced and suspended in 5 vol of 50 mM Tris·HCl buffer, pH 7.7, containing 25 mM KCl, 2.5 mM $MgCl_2$, 3 mM dithiothreitol (TKMD buffer), and 0.88 M sucrose. After homogenization, the microsomal membrane fraction was sedimented (100,000 × *g*) between 1.5 M and 2 M sucrose and stored at −20°C in 20 mM Hepes buffer, pH 7.5, containing 30% (vol/vol) glycerol.

Solubilization of Microsomal Membranes. Oviduct membranes (6 mg of protein) were suspended in 2 ml of 50 mM Tris·HCl, pH 7.5, containing 25 mM NaCl, 10 mM $MgCl_2$, 20 mM $MnCl_2$, 140 mM sucrose, and 0.2% Nonidet P40 (NP40) (Tris/NP40 buffer) and stirred for 3 hr at 4°C. The suspension was centrifuged at 250,000 × *g* for 15 hr and the supernatant fluid (crude soluble oligosaccharyltransferase) obtained was tested for its ability to catalyze the transfer of oligosaccharide from oligosaccharide-lipid to acceptor peptide.

Isolation of Oligosaccharide-Lipid. [^{3}H]Glucose-labeled oligosaccharide-lipid [oligo(Ose)-*PP*-Dol] was prepared by following the procedure of Spiro *et al.* (10) with modifications as follows: Standard assay mixtures were incubated in 10-ml test tubes at 37°C for 20 min in 50 mM Tris·HCl, pH 7.5, containing 2 μM UDP-GlcNAc, 2 μM GDP-Man, 125 nCi of UDP-[^{3}H]glucose (≈150,000 cpm), 0.4 mM $MgCl_2$, and 65 μl of oviduct membranes (0.4 mg of protein) in a final volume of 100 μl. The reaction was stopped by mixing with 1 ml chloroform/methanol (3:2, vol/vol). After addition of 200 μl of 4 mM $MgCl_2$ and 750 μg of bovine serum albumin (carrier protein), the mixture was centrifuged in a bench-top centrifuge for 5 min. The upper liquid phase was removed and the insoluble material at the interface was collected. This material was then extracted with 1 ml of chloroform/methanol (3:2) containing 0.7 mM $MgCl_2$ and centrifuged for 5 min. The resultant pellet was washed two more times with 1 ml of methanol/4 mM $MgCl_2$ (1:1, vol/vol), followed by centrifugation for 5 min. The [^{3}H]glucose-labeled oligosaccharide-lipid was isolated by extraction of the pellet two times with chloroform/methanol/water (1:1:0.3, vol/vol) (11). An aliquot was dried at 70°C and its radioactivity was measured in toluene scintillation fluid for estimation of oligosaccharide-lipid formed. When analyzed by thin-layer chromatography, this preparation migrated as a single radioactive species corresponding to oligo(Ose)-*PP*-Dol (12). Further, mild acid hydrolysis of the lipid yielded a single water-soluble compound whose chromatographic properties corresponded to those of an oligosaccharide greater than 2000 daltons. Unlabeled oligo(Ose)-*PP*-Dol was extracted from crude microsomal membranes by a similar fractionation procedure and purified on a column of DEAE-cellulose (13). The oligo(Ose)-*PP*-Dol was generously supplied by Patrick D. Reibold and Kathleen A. Presper of this laboratory.

Assay of Oligosaccharyltransferase Activity. The standard incubation was conducted in a 2-ml polyethylene tube in a total volume of 75 μl at pH 7.5. In a typical experiment either [^{3}H]glucose-labeled oligo(Ose)-*PP*-Dol (30,000 cpm) or unlabeled oligo(Ose)-*PP*-Dol (0.75 nmol) was dried under N_2 and dispersed in 50 mM Tris·HCl, pH 7.5, containing 25 mM NaCl, 20 mM $MnCl_2$, 140 mM sucrose, and 0.2% NP40 (TSMS/NP40 buffer). ^{125}I-Labeled peptide acceptor and enzyme were added and the mixture was incubated at 37°C for the periods indicated. The reaction was stopped by addition of an equal volume of buffer and chilling the mixture in an ice bath. Control incubation mixtures contained the complete system minus either oligo(Ose)-*PP*-Dol or enzyme.

A suspension of Con A-agarose in 1 M NaCl (100 μl; 9:1, vol/vol) was mixed with an equal volume of 20 mM Tris·HCl, pH 7.5, containing 0.2 M NaSCN, 1 mM $MnCl_2$, 1 mM $CaCl_2$ (TSMC buffer), and bovine serum albumin at 5 mg/ml. The mixture was mixed vigorously and allowed to stand at room temperature for 4–5 min. An aliquot of the incubation mixture (30 μl) was mixed with the gel slurry and allowed to stand at room temperature for 5 min. The slurry was then transferred to a Nuflow cellulose acetate membrane filter mounted on an aspirator and washed extensively with 30 ml of TSMC buffer containing 0.1% NP40. The washed filters were carefully removed from the support to avoid loss of resin and then transferred to polyethylene vials for ^{125}I radioactivity determination.

Incubation mixtures containing unlabeled peptide acceptor and [^{3}H]glucose-labeled oligo(Ose)-*PP*-Dol were prepared and incubated in an identical manner. However, the ^{3}H-labeled product was determined by fractionation of aliquots of the mixtures on a Sephadex G-50 column, equilibrated with 20% (vol/vol) acetic acid; the ^{3}H-labeled glycopeptide was eluted from the column with the same solvent.

Gel Filtration Chromatography. Gel filtration was performed on a column (1 × 52 cm) containing Sephadex G-50 equilibrated with either 0.1 M NH_4HCO_3 or 20% acetic acid. The void volume (V_o) of the column was determined with blue dextran and the retention volume (V_r) by phenol red.

Con A Affinity Chromatography. Con A-derivatized agarose was transferred to a 5-ml pipette in a packed volume of 2 ml and washed extensively with TSMC buffer containing 0.1% NP40. An aliquot of the incubation mixture was applied to the column and allowed to penetrate the resin, and the column was allowed to stand for 10 min before fractionation was continued. The column was then washed with a sufficient volume of TSMC buffer containing 0.1% NP40 to reduce the amount of radioactivity in the eluate to the basal level. Adsorbed material was then eluted with TSMC buffer containing 0.1% NP40 and 1% α-methyl mannoside (αMeMan); 1-ml fractions were collected and an aliquot from each fraction was analyzed for total content of ^{125}I-labeled glycopeptide.

Treatment of Glycopeptides with Endo-β-*N*-acetylglucosaminidase *H*. ^{125}I-Labeled glycopeptide fractions obtained from the Con A-agarose column were pooled, lyophilized, and dissolved in a minimal volume of 50 mM Tris·HCl, pH 7.5. To remove αMeMan, the solution was fractionated on a Sephadex G-50 column and the ^{125}I-labeled glycopeptide fractions were collected, lyophilized, dissolved in 200 μl of 50 mM Tris·HCl at pH 7.5, and finally analyzed for their susceptibility to degradation by endo-β-*N*-acetylglucosaminidase *H* as follows: An aliquot of the solution was adjusted to pH 6 by the addition of 0.3 M sodium citrate, pH 5.5; 2 milliunits of endo-β-*N*-acetylglucosaminidase *H* was added, and the mixture was incubated at 37°C for 18 hr in a toluene atmosphere. For control experiments, an equivalent amount of endo-β-*N*-acetylglucosaminidase *H* was heated at 100°C for 5 min before addition to the glycopeptide solution. Both control and endo-β-*N*-acetylglucosaminidase *H*-treated glycopeptides were reanalyzed by chromatography on a Con A-agarose column as described above.

Pronase Digestion of Glycopeptides. [^{3}H]Glucose-labeled glycopeptide fractions obtained from a Sephadex G-50 column were pooled, lyophilized, and dissolved in a minimal volume of 50 mM Tris·HCl, pH 7.5. To 100-μl aliquots of this solution $CaCl_2$ was added to a final concentration of 15 mM, followed by the addition of 500 μg of Pronase. The mixture was incubated with toluene at 37°C for 2 days with an intermediate addition of another 500 μg of Pronase. The mixture was then analyzed by Sephadex G-50 chromatography.

Measurement of Radioactivity. ^{3}H radioactivity was de-

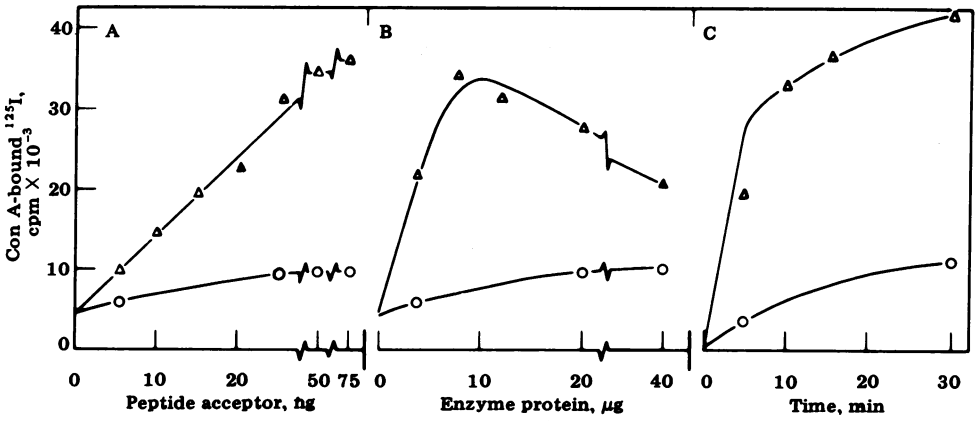

FIG. 1. Oligosaccharyltransferase activity in crude solubilized preparation. Rate of formation of Con A-agarose-bound ^{125}I-glycopeptide as a function of: acceptor peptide concentration (*A*); enzyme concentration (*B*); and time (*C*). ^{125}I-Peptide acceptor and oligo(Ose)-*PP*-Dol were incubated with the 250,000 × *g* crude supernatant fluid for 30 min except in *C*. Δ, Experimental samples; O, controls [minus enzyme or minus oligo(Ose)-*PP*-Dol].

termined in a Beckman model LS 3133T scintillation spectrometer after dissolving samples in Soluene 350 and a toluene-based scintillation fluid. ^{125}I radioactivity was measured by placing samples directly into a Beckman Biogamma II counting system.

Analytical Methods. Protein was determined by the method of Lowry *et al.* (14), using chicken ovalbumin as standard; samples were dialyzed to remove sucrose and M^{2+} before the protein estimation was performed. Lipid phosphate was determined according to Chen *et al.* (15) as modified by Ames and Dubin (16). Amino acid analysis was performed in Femto buffer, pH 2.0 (Durrum Chemical, Sunnyvale, CA) in a narrow-bore amino acid analyzer after hydrolyzing the sample in 6 M HCl for 24 hr.

RESULTS AND DISCUSSION

Soluble Oligosaccharyltransferase Activity. Assay of the 250,000 × *g* supernatant fluid obtained after centrifugation of detergent-treated microsomal membranes indicated that virtually all of the oligosaccharyltransferase activity had been solubilized by this procedure. Assay of the 250,000 × *g* pellet under a variety of conditions indicated that less than 5% of the total activity could be detected in this fraction. As shown in Fig. 1, the binding of radioactive iodine to Con A-agarose was proportional to the amount of added ^{125}I-peptide acceptor up to 633 ng/ml (*A*), whereas only limited proportionality to enzyme concentration (*B*) and time of incubation (*C*) was demonstrated. The detailed explanation for this lack of proportionality with time of incubation and enzyme concentration has not yet been determined.

The ^{125}I-labeled Con A-agarose-bound product formed by the crude soluble oligosaccharyltransferase preparation was isolated preparatively from a Con A-agarose column and tentatively characterized as the glycosylated form of ^{125}I-α-lactalbumin-(29–58) by its behavior on gel filtration columns and by its loss of affinity for Con A-agarose after treatment with endo-β-*N*-acetylglucosaminidase *H* (data not shown).

Purification of Oligosaccharyltransferase. On the basis of our results obtained with crude solubilized oligosaccharyltransferase preparations, we reasoned that, because a peptide derived from bovine α-lactalbumin is an acceptor for the transferase, α-lactalbumin-derivatized Sepharose may serve as an affinity resin for purification of the enzyme. Preliminary experiments indicated indeed that oligosaccharyltransferase activity was quantitatively adsorbed when the crude 250,000 × *g* supernate was passed over a column containing α-lactalbumin-Sepharose. A variety of approaches to recover the transferase activity from the gel included washing the column with divalent cation-free enzyme buffer (with and without 5 mM EDTA or 0.5 M KCl). These experiments indicated that oligosaccharyltransferase activity is quantitatively eluted from the gel with divalent cation-free buffer; the presence of either EDTA or KCl did not affect the overall recovery of activity. Therefore, oligosaccharyltransferase was purified on a large scale as follows: A 5-ml solution of solubilized activity (15 mg of protein) was applied to a α-lactalbumin-Sepharose column (0.8 × 4.5 cm); the column was washed with 11 column volumes of Tris/NP40 buffer until the A_{660} of the eluate in the protein assay (14) was baseline; and, finally, the column was washed with 7 ml of divalent cation-free Tris/NP40 buffer. As shown in Fig. 2, assay of fractions eluted from this column indicated that the transferase activity was quantitatively bound to the resin and eluted in the metal-free buffer solution. The fractions (13–16) that exhibited enzyme activity were pooled, and determination of the specific activity of oligosaccharyltransferase indicated a purification of approximately 2000-fold, relative to the specific activity of the enzyme in microsomal membranes. The long-term stability of purified oligosaccharyltransferase has not yet been determined; however, storage of the preparation (160 μg of protein per ml) at 4°C for periods up to 1 week resulted in less than 20% decrease in specific activity.

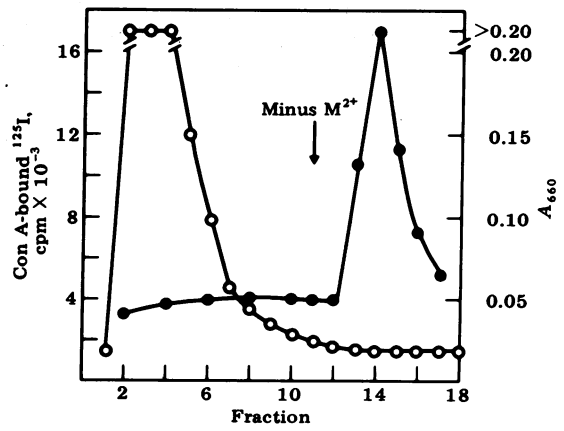

FIG. 2. Purification of oligosaccharyltransferase on an α-lactalbumin-Sepharose column. The crude solubilized preparation was fractionated as described in the text. O, Protein (14); ●, transferase activity.

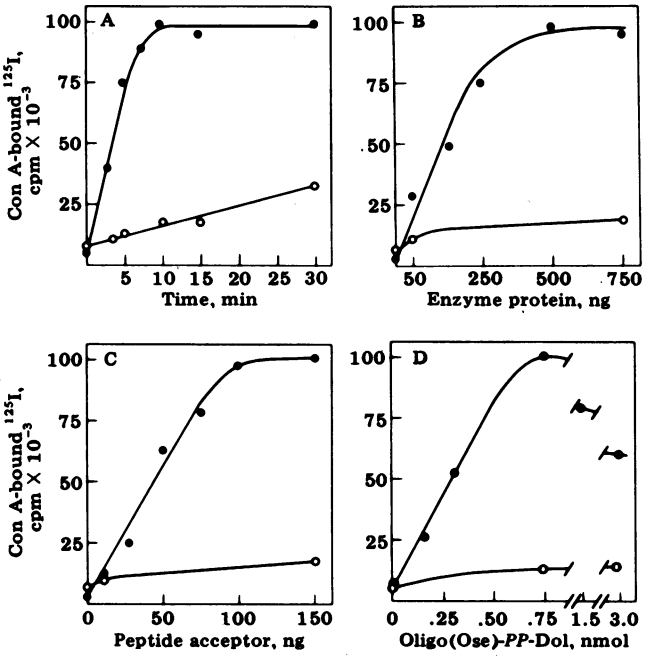

FIG. 3. Properties of purified oligosaccharyltransferase. (*A*) Time of incubation; (*B*) enzyme concentration; (*C*) peptide acceptor concentration; and (*D*) oligo(Ose)-*PP*-Dol concentration. Incubation was conducted for 10 min except in *A*. ●, Experimental samples; ○, controls (minus enzyme or minus oligo(Ose)-*PP*-Dol).

Properties of Oligosaccharyltransferase. The activity of oligosaccharyltransferase over the pH range 4–8 was maximal between pH 7 and 7.5, with approximately 30% and 20% of maximal activity at pH 6 and pH 8, respectively. In Fig. 3, the relationship of the transferase activity to time of incubation (*A*), enzyme concentration (*B*), peptide acceptor concentration (*C*), and oligo(Ose)-*PP*-Dol concentration (*D*) are illustrated. The assay procedure employed indicates that glycopeptide formation is proportional to the time of incubation for periods up to 10–15 min; extended incubation of the assay system beyond this period resulted in variable proportionality of the activity in spite of the fact that approximately 5% of the substrates had been utilized. Mild acid hydrolysis of oligo(Ose)-*PP*-Dol resulted in total loss of oligosaccharyltransferase activity. The formation of glycopeptide is directly proportional to the amount of enzyme used as well as to the concentration of oligo(Ose)-*PP*-Dol and ^{125}I-peptide acceptor. Under the assay conditions employed, an apparent saturating concentration of ^{125}I-peptide is 0.33 μM, whereas the apparent saturating concentration of oligo(Ose)-*PP*-Dol is 10 μM.

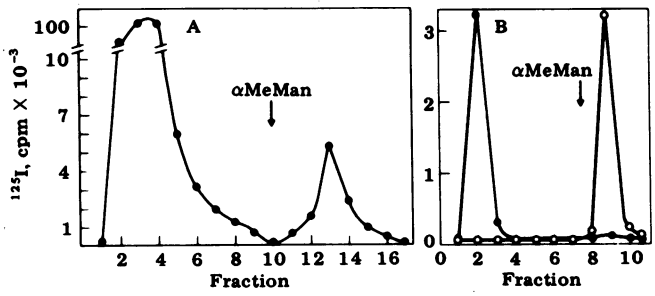

FIG. 4. Con A-agarose column chromatography of enzymatically prepared ^{125}I-glycopeptide before and after treatment with endo-β-*N*-acetylglucosaminidase *H*. (*A*) A large-scale incubation mixture (0.75 ml) was fractionated on a column (2 ml) of Con A-agarose as described in the text; 1-ml fractions were collected and the radioactivity contained in 10-μl aliquots is plotted. (*B*) The glycopeptide fractions isolated from the Con A-agarose column in *A* were pooled and chromatographed on Sephadex G-50 to remove αMeMan; fractions containing ^{125}I-glycopeptide were pooled, lyophilized, and treated with endo-β-*N*-acetylglucosaminidase *H* as described in the text. The glycosidase-treated sample (●) and a control sample (○) treated in an identical manner with heat-inactivated glycosidase were rechromatographed on the Con A-agarose column; 1-ml fractions were collected and their radioactivities were measured.

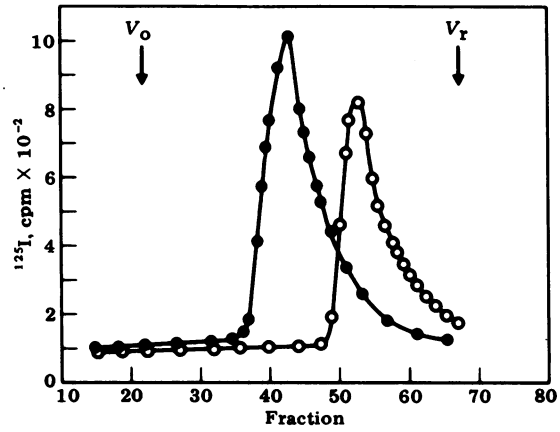

FIG. 5. Comparison of enzymatically synthesized ^{125}I-glycopeptide and ^{125}I-peptide acceptor on Sephadex G-50. An aliquot of the ^{125}I-glycopeptide obtained from Con A-agarose column as described in Fig. 4*A* and an equivalent amount of ^{125}I-peptide acceptor were fractionated separately on a column (1 × 52 cm) of Sephadex G-50 as described in the text; 750-μl fractions were collected. V_o, void volume; V_r, retention volume; ●, ^{125}I-glycopeptide; ○, ^{125}I-peptide acceptor.

Assay of purified oligosaccharyltransferase in the absence of divalent cation resulted in the detection of insignificant activity, whereas inclusion of 3 mM Mn^{2+} in the incubation mixture resulted in maximal activity of the transferase; higher concentrations of Mn^{2+} (up to 12 mM) did not significantly affect the enzymatic activity. In the presence of 3 mM Mn^{2+}, addition of EDTA resulted in inhibition of transferase activity directly proportional to the concentration of EDTA in the incubation mixture. Substitution of Mg^{2+} for Mn^{2+} resulted in partial recovery of transferase activity; at a concentration of 3 mM Mg^{2+} the enzyme exhibited approximately 30% of the maximal activity observed in the presence of Mn^{2+}, and higher concentrations markedly inhibited the enzyme.

The radioactive product of the oligosaccharyltransferase reaction catalyzed by the purified enzyme was characterized as an ^{125}I-labeled glycopeptide by a variety of techniques. A large-scale incubation mixture was prepared by combining ten assay mixtures (total volume 0.75 ml) and, after dilution, the mixture was fractionated on a column containing Con A-agarose* as illustrated in Fig. 4*A*. An aliquot of the radioactive material (≈5000 cpm) eluted from the Con A-agarose gel with αMeMan was treated by gel filtration to remove αMeMan, digested with endo-β-*N*-acetylglucosaminidase *H*, and reapplied to a column containing Con A-agarose. As shown in Fig. 4*B*, exhaustive digestion with the specific endoglycosidase resulted in complete loss of affinity for the lectin-derivatized affinity resin, indicating that a glycosylamine-linked carbohydrate side chain had been cleaved from the peptide.

A second aliquot of the radioactive material eluted with αMeMan from the Con A-agarose column was fractionated on a column of Sephadex G-50 as illustrated in Fig. 5. The radioactive glycopeptide fractionated on the column as a molecule

* Application of this preparation to a column containing ricin-derivatized Sepharose resulted in quantitative recovery of the radioactive product in the unadsorbed void volume eluate.

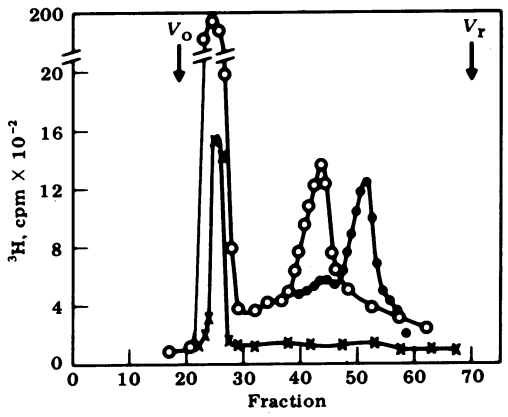

FIG. 6. Protease digestion of ^{3}H-labeled glycopeptide. An incubation mixture containing [^{3}H]glucose-labeled oligo(Ose)-*PP*-Dol and unlabeled peptide acceptor was prepared as described in the text and fractionated on a column (1 × 52 cm) of Sephadex G-50 (○); a control mixture (minus peptide acceptor) was fractionated separately on the same column (×). The fractions (37–47) containing ^{3}H-labeled glycopeptide were isolated, treated with Pronase, and rechromatographed on the column (●). Fractions (750 μl) were collected on an automatic collector.

of somewhat higher molecular weight than the nonglycosylated ^{125}I-labeled peptide acceptor. Estimates of the molecular mass differences between these two peptides suggest that the glycopeptide synthesized in the oligosaccharyltransferase-catalyzed reaction is approximately 2500 daltons greater than that of the nonglycosylated peptide acceptor (≈4000 daltons); this difference in mass may be accounted for by transfer of an oligosaccharide consisting of 12–14 GlcNAc, Man, and Glc residues to the peptide in the reaction.

In order to confirm the glycopeptide structure of the product of the transferase, an incubation mixture was prepared containing unlabeled peptide acceptor and [^{3}H]glucose-labeled oligo(Ose)-*PP*-Dol. After incubation under standard conditions, the entire mixture and a control mixture (complete system minus enzyme) were fractionated on a column containing Sephadex G-50 as shown in Fig. 6. Two radioactive components were resolved in the sample of the incubation mixture; a ^{3}H-labeled component corresponding to the position (fractions 37–47) of the ^{125}I-glycopeptide (see Fig. 5) and a larger quantity of ^{3}H-labeled material at the void volume of the column (fractions 21–29). Conversely, fractionation of the control mixture indicated the presence of only the ^{3}H-labeled material emerging in the void volume of the column. All of the radioactivity present in the void volume material may be accounted for as [^{3}H]glucose-labeled oligo(Ose)-*PP*-Dol, suggesting that the oligosaccharide-polyprenol is organized in a micellar structure under these conditions. The ^{3}H-labeled material contained in fractions 37–47 was isolated, exhaustively digested with Pronase, and rerun on the same column of Sephadex G-50; the radioactive product of this treatment fractionated as a compound of significantly lower molecular weight. In addition, behavior of the Pronase-treated compound on paper electrophoresis was consistent with that of a glycosylamine-linked glycopeptide obtained by treatment of standard glycoproteins with Pronase.

On the basis of these results, we have concluded that the purified soluble oligosaccharyltransferase catalyzes the following reaction:

$$\text{oligo(Ose)-}PP\text{-Dol} + {}^{125}\text{I-peptide} \rightarrow \text{oligo(Ose)-}{}^{125}\text{I-peptide} [+ PP\text{-Dol}].$$

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