Purification and Characterization of Dolichyl-P-mannose:$Man_5(GlcNAc)_2$-PP-dolichol Mannosyltransferase†

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ABSTRACT: The dolichyl-P-mannose:dolichyl-PP-heptasaccharide α-mannosyltransferase (2.4.1.130), which catalyzes the transfer of mannose from dolichyl-P-mannose to the $Man_5(GlcNAc)_2$-PP-dolichol acceptor glycolipid, was solubilized from pig aorta microsomes with 0.5% NP-40 and purified 985-fold by a variety of conventional methods. The partially purified enzyme had a pH optimum of 6.5 and required Ca^{2+}, at an optimum concentration of 8–10 mM, for activity. Mn^{2+} was only 20% as effective as Ca^{2+}, and Mg^{2+} was inhibitory. The mannosyltransferase activity was also inhibited by the addition of EDTA to the enzyme, but this inhibition was fully reversible by the addition of Ca^{2+}. The enzyme was quite specific for dolichyl-P-mannose as the mannosyl donor and $Man_5(GlcNAc)_2$-PP-dolichol as the mannosyl acceptor. The K_m values for dolichyl-P-mannose and the acceptor lipid $Man_5(GlcNAc)_2$-PP-dolichol were 1.8 and 1.6 μM. On Bio-Gel P-4 columns and by HPLC, the radiolabeled oligosaccharide formed during incubation of dolichyl-P-[^{14}C]mannose and unlabeled $Man_5(GlcNAc)_2$-PP-dolichol with the purified enzyme behaved like $Man_6(GlcNAc)_2$. This octasaccharide was susceptible to digestion by endoglucosaminidase H, indicating that the newly added mannose was attached to the 6-linked mannose in an α1,3-linkage. This linkage was further confirmed by acetolysis of the oligosaccharide product [i.e., $Man_6(GlcNAc)_2$], which gave a labeled disaccharide as the major product (>90%).

The asparagine-linked oligosaccharide chains of most eucaryotic glycoproteins are derived from a common precursor, $Glc_3Man_9(GlcNAc)_2$-PP-dolichol (Kornfeld & Kornfeld, 1985; Kukuruzinska et al., 1987; Tanner & Lehle, 1987; Elbein, 1979), which is assembled in the endoplasmic reticulum by the stepwise addition of monosaccharides to the lipid carrier dolichyl-P (Struck & Lennarz, 1980; Hubbard & Robbins, 1980; Snider, 1984; Perez & Hirschberg, 1986; Spiro & Spiro, 1985). While some of the enzymes of this pathway have been partially purified and characterized from several sources (Plouhar & Bretthaur, 1983; Kaushal & Elbein, 1985; Haselbeck, 1989; Sharma et al., 1982; Jensen & Schutzbach, 1981; Schutzbach et al., 1980; Gold & Green, 1983), only the GlcNAc-1-P transferase has been reported to have been purified to homogeneity (Shailubhai et al., 1988). Furthermore, detailed emzymology and especially regulation of the dolichol cycle remain to be elucidated. The problem with purifying these enzymes lies in the difficulties of obtaining pure lipid-linked saccharides that can be used as acceptors of the sugars and also in the fact that most, if not all, of these membrane-bound glycosyltransferases are very labile. However, it does seem likely that the "dolichol pathway" is under some type of physiological control, and this control may be important in the regulation of N-linked glycoprotein biosynthesis. In order to understand this control, it will be necessary to purify and characterize the various enzymes in the pathway.

As a result of the above considerations, we decided to study the mannosyltransferase that adds the sixth mannose to the lipid-linked oligosaccharides. This enzyme seemed to us to be of particular interest since this is the step where there is a shift in mannosyl donor from GDP-mannose to dolichyl-P-mannose. In this paper, we report the partial purification of this enzyme from pig aorta microsomes and some of the properties of this mannosyltransferase. This enzyme adds the sixth mannose, from dolichyl-P-mannose, to the 6-linked mannose of $Man_5(GlcNAc)_2$-PP-dolichol in an α1,3-linkage to produce Manα1,2Manα1,2Manα1,3[Manα1,3Manα1,6]-Manβ1,4GlcNAc-GlcNAc.

Experimental Procedures

Materials. GDP-[^{14}C]mannose (253 mCi/mmol), UDP-[6-^{3}H]GlcNAc (18 Ci/mmol), and NaB^3H_4 (528 mCi/mmol) were purchased from New England Nuclear Co. Dolichyl phosphate, nucleoside diphosphate sugars, and Nonidet P-40 were from Sigma Chemical Co. Hydroxyapatite, Bio-Gel P-4 (200–400 mesh), β-mercaptoethanol, AG 50-W×8 (H^+), and AG 1×8 ($HCOO^-$) were purchased from Bio-Rad Laboratories. DEAE-cellulose (DE-52) was from Whatman Chemical Separations, Ltd. Sephacryl S-300 and Sephadex LH-20 were supplied by Pharmacia Co. Endo-β-*N*-acetylglucosaminidase H (Endo H) from *Streptomyces plicatus* was obtained from Miles Laboratories, and Micropak Ax-5 HPLC columns were purchased from Varian Associates. All other chemicals were obtained from reliable chemical sources and were of the best grade available. Fresh pig aorta was obtained from the local slaughterhouse and was processed immediately to prepare microsomes.

Preparation of Particulate Enzyme from Pig Aorta. All operations were done at 0–5 °C. The intimal layer from fresh pig aorta (approximately 375 g of tissue) was homogenized in 2 L of 50 mM Tris buffer, pH 7.4, in a Waring blender. The homogenate was filtered through cheesecloth and centrifuged at 12000*g* for 10 min. The supernatant fluid was collected and centrifuged at 100000*g* for 90 min. The supernatant fluid from this centrifugation was discarded, and the microsomal pellet was suspended in 50 mM HEPES buffer, pH 6.5, containing 10% glycerol and 0.1% β-mercaptoethanol to obtain a protein concentration of 10 mg/mL. This membrane suspension was used for enzyme purification studies and is referred to as the crude microsomal fraction. For preparation of the lipid-linked saccharide substrates, microsomes

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were suspended in 50 mM Tris-HCl buffer, pH 7.4.

Solubilization of the Mannosyltransferase. To 50 mL of microsomal fraction (500 mg of protein) was added dropwise, with gentle stirring, 2.5 mL of 10% (w/v) Nonidet P-40, so that the final detergent concentration was 0.5% and the protein:detergent ratio was 2:1. The enzyme was solubilized by gentle stirring for 20 min in ice, followed by centrifugation at 100000*g* for 60 min to remove the insoluble material. The supernatant liquid, which contained the solubilized mannosyltransferase, was carefully removed with the use of a Pasteur pipet, and additional prechilled glycerol was added to attain a final concentration of 20%. This addition of glycerol was necessary to stabilize the solubilized enzyme preparation. This enzyme preparation was fully active for up to 3 months when stored at −20 °C and for about 96 h at 0 °C.

Preparation and Purification of Lipid-Linked Saccharide Substrates. Dolichyl-P-[^{14}C]mannose was prepared by incubating GDP-[^{14}C]mannose with dolichyl-P and pig aorta microsomes (Chambers et al., 1977). For large-scale preparations, the procedure was as follows: 24 tubes, each containing 200 μg of dolichyl-P, 80 μmol of Mg-EDTA, 0.1% Nonidet P-40, 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 4 μmol of GDP-[^{14}C]mannose (sp act. 253 mCi/mmol), and membrane preparation (8 mg of protein) in a final volume of 4 mL, were incubated at 37 °C for 30 min. The reaction was terminated by the addition of 4 mL of $CHCl_3$ and 4 mL of CH_3OH, and the mixture was vortexed vigorously. The lower or $CHCl_3$ layer contained the dolichyl-P-mannose, and it was removed and treated as described previously (Chambers et al., 1977). The dolichyl-P-[^{14}C]mannose was purified by several thin-layer chromatographies, as well as by chromatography on DEAE-cellulose (Shailubhai et al., 1988). After these purifications, the radioactive lipid showed only a single radioactive spot on TLC plates in several different solvent systems. In addition, a single radioactive sugar, corresponding to mannose, was detected when the lipid was subjected to mild acid hydrolysis followed by either paper chromatography or by HPLC.

For the preparation of acceptor lipid $Man_5(GlcNAc)_2$-PP-dolichol, lipid-linked oligosaccharides were extracted and purified from pig liver (Kaushal & Elbein, 1986) and then incubated with a solubilized enzyme preparation from pig aorta in the presence of GDP-mannose to produce the $Man_5(GlcNAc)_2$-PP-dolichol. When EDTA or amphomycin (Chambers et al., 1977) was added to these incubations, the solubilized enzyme system was not able to synthesize dolichyl-P-mannose from GDP-mannose and, therefore, could not elongate lipid-linked oligosaccharides beyond the $Man_5(GlcNAc)_2$-PP-dolichol stage. Thus, the basic procedure for preparation of this acceptor lipid is given below. About 2 kg of pig liver was homogenized in a Waring blender in 2 L of distilled water, and the slurry was made 1:1:1 ($CHCl_3$–CH_3OH–H_2O) by the addition of $CHCl_3$ and CH_3OH. The suspension was mixed vigorously, and the phases were allowed to separate. The lower, $CHCl_3$ phase was removed and discarded, and the upper phase and interface were removed and suspended in 5 L of 10:10:3 $CHCl_3$:CH_3OH:H_2O to extract the lipid-linked oligosaccharides. After being allowed to stand overnight at room temperature, the residue was removed by filtration or centrifugation, and the filtrate was concentrated to dryness and resuspended in the 10:10:3 solvent. The lipid-linked oligosaccharides were purified by repeated chromatography (three times) on columns of DEAE-cellulose in $CHCl_3$–CH_3OH–H_2O, and the charged lipids were eluted with gradients of ammonium formate (0–200 mM). Phospholipids were removed by saponification followed by DEAE-cellulose chromatography (Kaushal & Elbein, 1986).

Aliquots from each fraction of the DEAE-cellulose chromatography were assayed for their ability to serve as acceptors of mannose from GDP-[^{14}C]mannose into lipid-linked oligosaccharides by a solubilized enzyme preparation of pig aorta. Fractions that stimulated mannose incorporation into lipid-linked oligosaccharides were pooled and partitioned into chloroform. The chloroform was removed under reduced pressure, and the lipid was suspended in a small volume of 0.1% NP-40 and incubated with unlabeled GDP-mannose, EDTA, and the solubilized enzyme fraction from pig aorta. The purpose of this incubation was to elongate any smaller sized lipid-linked oligosaccharides to the $Man_5(GlcNAc)_2$-PP-dolichol stage. After incubation, the lipid-linked oligosaccharides were isolated by solvent extraction, saponified, and purified by chromatography on DEAE-cellulose. In some cases, a small amount of radiolabeled $Man_5(GlcNAc)_2$-PP-dolichol was added to help locate and identify the lipid-linked oligosaccharide. Identification of the oligosaccharide component of the acceptor lipid was done by hydrolysis and reduction with NaB^3H_4 as described below. In some cases, the lipid-linked oligosaccharide fraction was further purified by incubation in dilute detergent with endoglucosaminidase H (Chalifour & Spiro, 1984) to cleave any oligosaccharides containing six or more mannose residues. After incubation, the uncleaved lipid-linked oligosaccharides [mostly $Man_5(GlcNAc)_2$-PP-dolichol] were reisolated by chromatography on DEAE-cellulose columns.

Mild Acid Hydrolysis and Reduction with NaB^3H_4. Mild acid hydrolysis of the lipid-linked oligosaccharides was done in 50% propanol containing 0.02 N HCl at 100 °C for 20 min as previously described (Forsee & Elbein, 1975). The released oligosaccharides were concentrated to dryness several times under reduced pressure to remove the HCl and were neutralized with ammonia. The oligosaccharides were then reduced by the addition of NaB^3H_4 in 5 mM NaOH (Badet & Jeanloz, 1988). The mixture was allowed to stand overnight at room temperature and was then neutralized with acetic acid to destroy any remaining $NaBH_4$. The solution was passed through columns and Dowex 50 (H^+) to remove Na^+, the wash of the column was concentrated to dryness, and the borate was removed by repeated dissolution of the residue in methanol and removal of the solvent under reduced pressure. The ^{3}H-labeled oligosaccharides were then further purified from contaminating ^{3}H by paper chromatography (Endo et al., 1979). The radioactive oligosaccharide band which remained at or near the origin was cut from the paper and eluted with water. This oligosaccharide was then identified by chromatography on columns of Bio-Gel P-4 as well as by HPLC (Hirani et al., 1987).

Assay of Mannosyltransferase Activity. The standard incubation mixture that was used for assaying this enzyme contained the following components in a final volume of 0.4 mL: acceptor lipid (0.5 μM), dolichyl-P-[^{14}C]mannose (12000 cpm), 50 mM HEPES buffer, pH 6.5, NP-40 (0.1%), $CaCl_2$ (10 mM), β-mercaptoethanol (0.05%), and 10–200 μg of enzyme, depending on the state of purity of the enzyme and the experiment in question. Incubations were done for 20 min at 37 °C, unless otherwise stated. The reaction was terminated by the addition of 2 mL of 1:1 $CHCl_3$:CH_3OH and 0.6 mL of H_2O, and the mixture was vortexed vigorously. The phases were separated by centrifugation, and the lower $CHCl_3$ phase, containing the dolichyl-P-mannose, was removed and discarded. The upper phase and the interface were reextracted

with another 1-mL portion of chloroform, and again the lower phase was discarded. To the upper phase and interface was added 1 mL of CH_3OH to dissolve any remaining $CHCl_3$, and the particulate material, containing the lipid-linked oligosaccharides was isolated by centrifugation. The pellet was washed two times with 3:48:47 $CHCl_3$:CH_3OH:H_2O, and the lipid-linked oligosaccharides were then extracted by suspending the particulate material in 10:10:3 $CHCl_3$:CH_3OH:H_2O. After two extractions, each with 2 mL of this solvent, an aliquot of the 10:10:3 solution was transferred to scintillation vials for the determination of the amount of radioactivity transferred to lipid-linked oligosaccharides.

Product Analysis. In order to obtain a sufficient amount of product for characterization, incubation mixtures were scaled up at least 5 times, and the radiolabeled lipid-linked oligosaccharides were isolated and purified as described above. The oligosaccharides were released from the lipid by mild acid hydrolysis and were characterized by chromatography on a calibrated Bio-Gel P-4 column (1.5 × 150 cm) and by HPLC (using a Micropak AX-5 amino-bonded column). These columns were standardized with the following oligosaccharide standards: $Glc_3Man_9(GlcNAc)_2$, $Glc_2Man_9(GlcNAc)_2$, $Man_9(GlcNAc)_2$, $Man_8(GlcNAc)_2$, $Man_7(GlcNAc)_2$, $Man_6(GlcNAc)_2$, $Man_5(GlcNAc)_2$, and mannose. Various samples were also treated with endoglucosaminidase H to determine their susceptibility to this enzyme, and the products were analyzed by Bio-Gel P-4 chromatography.

Structural and Analytical Procedures. Digestion of acid-released oligosaccharides or the intact lipid-linked oligosaccharides with endoglucosaminidase H was done in 200 μL of 50 mM sodium citrate buffer, pH 5.5, containing 5–10 munits of enzyme at 37 °C for 24 h under a toluene atmosphere. When lipid-linked oligosaccharides were used as substrates, they were added to assay tubes in $CHCl_3$–CH_3OH, and the solvent was removed under a stream of nitrogen. The lipid was then suspended in 0.1% Triton X-100 before the other reaction components were added. Acetolysis of the radiolabeled oligosaccharide products was performed as previously described (Romero & Herscovics, 1986), and the acetolyzed products were identified by HPLC (Hirani et al., 1987) and by Bio-Gel P-4 chromatography (Kaushal & Elbein, 1986). TLC of lipid-linked saccharides was done on silica gel G plates in 10:10:3 $CHCl_3$:CH_3OH:H_2O (Badet & Jeanloz, 1988). Protein was measured by the method of Bradford (1976), and phosphorus was determined as described by Duck-Chong (1979). SDS gel electrophoresis was done in 12% gels as previously described (Laemmli, 1970).

Results

Identification of the Acceptor Lipid-Linked Saccharide. In order to purify and characterize the mannosyl transferase that adds the first mannose residue from dolichyl-P-mannose (i.e., the sixth mannose of the lipid-linked oligosaccharides), it was necessary to have the appropriate $Man_5(GlcNAc)_2$-PP-dolichol to serve as a mannose acceptor. As indicated under Experimental Procedures, this lipid-linked oligosaccharide was isolated and purified from pig liver, and the oligosaccharide was released from the purified lipid by mild acid hydrolysis and reduced with NaB^3H_4. The oligosaccharide alcohols were then analyzed by HPLC as shown in Figure 1. It can be seen that about 85% of the radioactivity emerged in a sharp, symmetrical peak in the same position as shown by the $Man_5(GlcNAc)_2$ standard synthesized in vitro in the presence of amphomycin or EDTA (Chambers et al., 1977). However, approximately 15% of the radioactivity was in a larger oligosaccharide that emerged in the same area as the $Man_6(GlcNAc)_2$ standard.

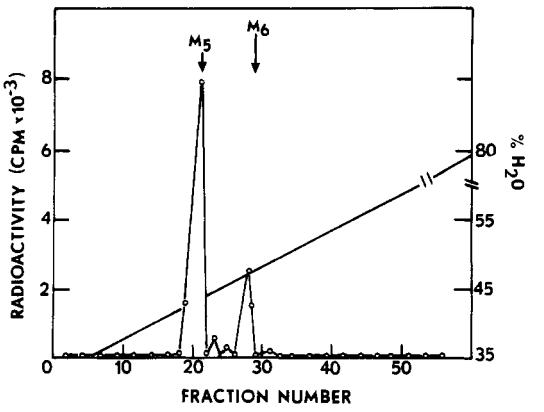

FIGURE 1: Identification of oligosaccharide portion of lipid-linked oligosaccharide by HPLC. The acceptor lipid was purified from pig liver as described. The oligosaccharide was released by mild acid hydrolysis, reduced with NaB^3H_4, and applied to a Micropak Ax-5 column that had been equilibrated with CH_3CN–H_2O (65:35). The column was eluted by increasing the H_2O by 0.5%/min for 90 min. The flow rate was 1 mL/min. Fractions of 1 mL were collected, and an aliquot was taken for the determination of radioactivity. Standards are $Man_5(GlcNAc)_2$ = M_5 and $Man_6(GlcNAc)_2$ = M_6.

The radioactive oligosaccharide that emerged in the $Man_5(GlcNAc)_2$ area of the column was resistant to the action of endoglucosaminidase H, indicating that it was Manα1,2Manα1,2Manα1,3(Manα1,6)Manβ1,4GlcNAc-GlcNAc. Thus, this lipid-linked oligosaccharide fraction was used as an acceptor in many of the reactions discussed below, even though it did contain small amounts of the $Man_6(GlcNAc)_2$-PP-dolichol. However, in some experiments this $Man_6(GlcNAc)_2$-PP-dolichol was removed by treating the entire lipid-linked oligosaccharide fraction with Endo H in order to cleave the larger lipid-linked oligosaccharides [i.e., $Man_6(GlcNAc)_2$-PP-dolichol and larger] and then reisolating the intact lipid-linked oligosaccharides by solvent fractionation. The results obtained with this purified mannosyl acceptor were generally similar to those with the less purified lipid acceptor.

Purification of the Mannosyltransferase. The results of the purification procedure for the mannosyltransferase are presented in Table I. The initial enzyme preparation was a microsomal fraction that sedimented at between 50000*g* and 105000*g*. The mannosyltransferase activity in this particulate material could be solubilized with 0.5% Nonidet P-40, and this solubilized activity was stable for at least 3 months when stored at −20 °C in the presence of 20% glycerol and 0.1% mercaptoethanol. All purification steps were done at 0–5 °C. The solubilized enzyme was brought to 50% saturation of $(NH_4)_2SO_4$, and the precipitate was collected and dissolved in 20 mL of 20 mM HEPES buffer, pH 6.5, containing 20% glycerol, 0.1% NP-40, and 0.1% β-mercaptoethanol. After dialysis overnight, the mannosyltransferase was applied to a column of DEAE-cellulose. Essentially all of the enzymatic activity emerged in the wash of the column, whereas a considerable amount of protein was retained, giving about a 2-fold purification (data not shown). About 50% of the applied mannosyltransferase activity was recovered from this column. The enzyme was next applied to a Sephacryl S-300 column as indiciated in Figure 2. This column resolved this activity into two peaks corresponding to apparant molecular masses of 80 and 85 kDa. Since peak II from this column had a higher specific activity and a much higher yield (Table I), this peak was used in further purification experiments. It is not clear whether the 80-kDa peak is a proteolytic degradation product of the 85-kDa protein or not. The enzyme at this stage was also reasonably stable and could be stored at −20 °C for several months with only slight loses in activity. A further

Table I: Purification of Mannosyltransferase from Pig Aorta

	total vol (mL)	total protein (mg)	enzyme act. (cpm/50 μL)	total enzyme act. (cpm × 10^{-3})	sp act. [cpm/(min·mg)]	purification (x-fold)	yield (%)
crude microsomes	120	1080	662	1588	73	1	
Nonidet-solubilized enzyme (105000*g* supermatant)	140	515	3106	8676[a]	842	11	100
$(NH_4)_2SO_4$ precipitation (0–50%)	45	224	6170	5553	1239	16	64
DE-52	24	72	6314	3030	2104	28	29
Sephacryl S-300							
peak I	10	5	1609	321	2925	39	3
peak II	13	17	3441	1858	5308	72	21
hydroxyapatite	9	0.2	1781	320	72840	985[b]	3

[a] Activation of mannosyltransferase by detergent during solubilization is evident. The activity of the solubilized enzyme was used as 100% for the purpose of estimating percent yield. [b] The actual purification factor would be higher, but for the unstable nature of the purified enzyme.

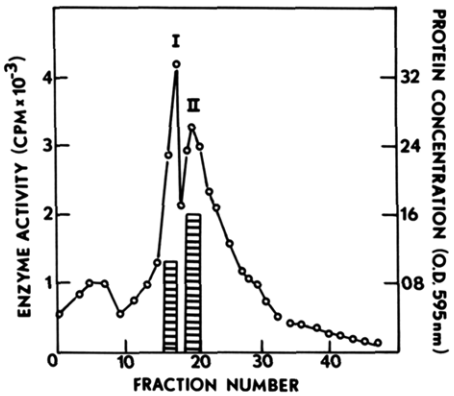

FIGURE 2: Purification of mannosyltransferase by gel filtration on Sephacryl S-300. The active fractions from the DE-52 column were concentrated to about 3 mL and applied to a column (2 × 54 cm) of Sephacryl S-300. The column was eluted with buffer, and fractions that were active for mannosyltransferase activity were pooled and saved.

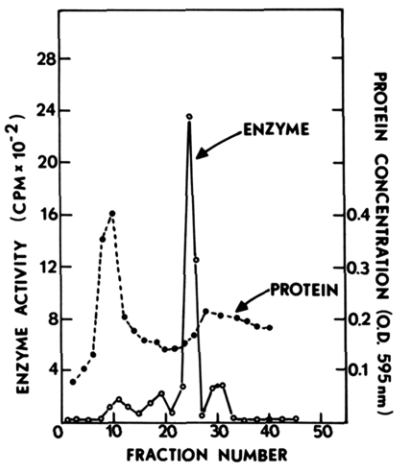

FIGURE 3: Purification of the mannosyltransferase by chromatography on hydroxyapatite. The active fractions from Sephracryl S-300 were pooled, concentrated to about 3 mL, adjusted to 10 mM with respect to potassium phosphate buffer, pH 6.7, and applied to a 1.5 × 9 cm column of hydroxyapatite. The column was washed with about 15 mL of the same buffer and then was eluted with a 100-mL linear gradient of 10–200 mM potassium phosphate buffer in the same buffer. Fractions of 1.5 mL were collected and tested for mannosyltransferase activity.

step in purification, as shown in Figure 3, was to apply the enzyme to a column of hydroxyapatite and elute the column with a gradient of phosphate buffer. The mannosyltransferase activity eluted in a sharp peak at about 130 mM phosphate buffer. Unfortunately, the enzyme was not very stable at this stage of purification and could only be kept for short periods of time (24 h in ice) before considerable loss of activity occurred. Attempts to stabilize the enzyme by the addition of

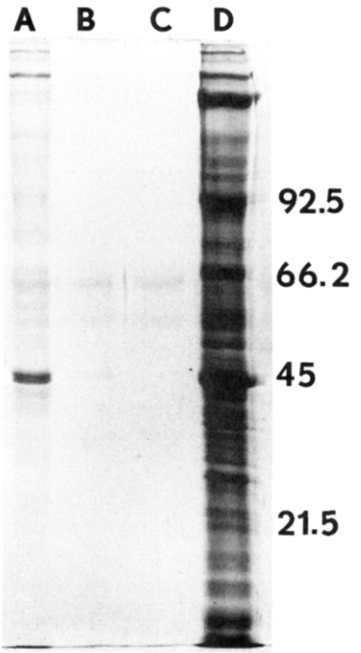

FIGURE 4: SDS gel electrophoresis of mannosyltransferase fractions: lane A = Sephracryl S-300 fraction; lanes B and C = hydroxyapatite fractions; lane D = solubilized enzyme fraction. Standard protein markers are shown at the right as follows: 92.5-kDa protein = phosphorylase B; 66.2-kDa protein = bovine serum albumin; 45-kDa protein = ovalbumin; 21.5-kDa protein = soybean trypsin inhibitor.

glycerol, β-mercaptoethanol, phospholipids, etc. were not successful. As a result, it was necessary to use the enzyme in various experiments immediately after elution from this column. The overall purification of the enzyme through these steps was about 980-fold, and the yield of active enzyme was about 4%. As seen in Figure 4, when the enzyme fraction from hydroxyapatite was subjected to SDS gel electrophoresis, it showed two major protein bands (lanes B and C) with molecular masses of 64.8 and 55 kDa. Also shown in this figure for comparison are SDS gel profiles of the solubilized enzyme fraction (lane D) and the preparation from Sephacryl S-300 (lane A).

Identification of the Reaction Product. In order to identify the product of the reaction, the assay was scaled up 5 times, and the radioactive lipid-linked oligosaccharide product was isolated as described under Experimental Procedures. The lipid-linked oligosaccharide was first run on thin-layer plates in $CHCl_3$–CH_3OH–H_2O as shown in Figure 5. It can be seen that the peak of radioactivity was separated from the acceptor lipid [i.e., $Man_5(GlcNAc)_2$-PP-dolichol] and from dolichyl-P-mannose. There was also a small amount of radioactivity in the dolichyl-P-mannose area of the TLC which is probably due to a carryover of some of the substrate during the ex-

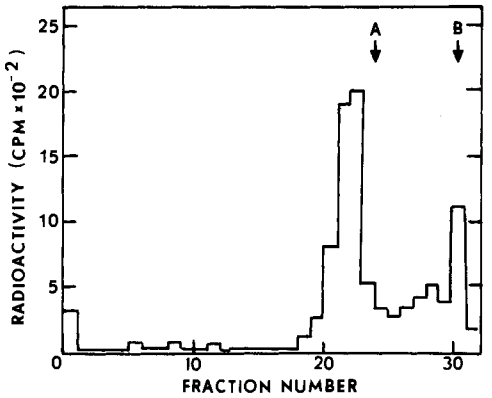

FIGURE 5: Identification of enzymatic reaction product by TLC. The product was isolated from incubation mixtures by solvent extraction, applied to silica gel G plates, and chromatographed in $CHCl_3$–CH_3OH–H_2O (10:10:3). The plate was dried, cut into sections, and counted in the scintillation counter to locate the radioactive bands. The arrows at the top indicate the locations of the standards: $Man_5(GlcNAc)_2$-PP-dolichol (A) and dolichyl-P-mannose (B).

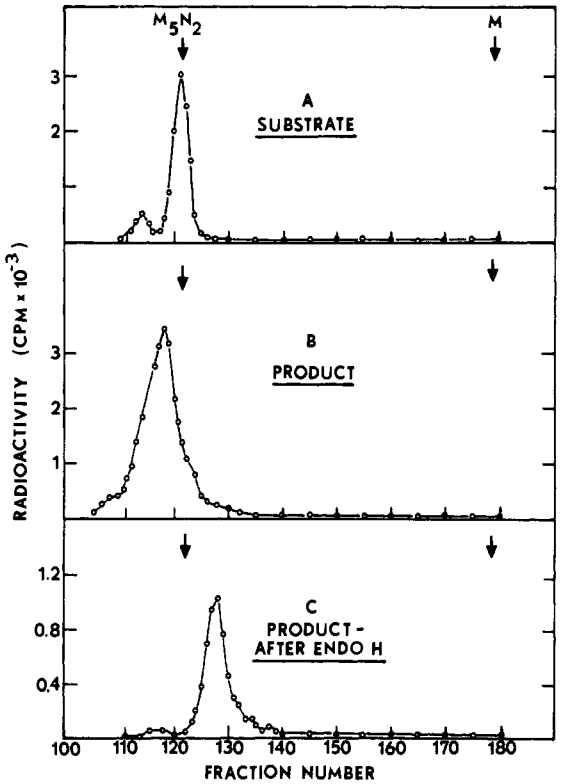

FIGURE 6: Identification of reaction product by chromatography on columns of Bio-Gel P-4. The radiolabeled lipid-linked oligosaccharide produced from dolichyl-P-[^{14}C]mannose and the partially purified mannosyltransferase was isolated by solvent extraction and subjected to mild acid hydrolysis, and the oligosaccharide was identified by Bio-Gel P-4 chromatography as seen in profile B. This oligosaccharide was also treated with endoglucosaminidase H to determine its susceptibility as shown by profile C. For comparison, the oligosaccharide from the acceptor lipid-linked oligosaccharide was reduced with NaB^3H_4 and run on the column. The standards shown at the top are $Man_5(GlcNAc)_2$ prepared by incubation of liver microsomes with GDP-[^{14}C]mannose in the presence of amphomycin.

traction. The radiolabeled lipid-linked oligosaccharide was subjected to mild acid hydrolysis, and the liberated oligosaccharide was identified by gel filtration as demonstrated in Figure 6. The upper profile shows the migration of the oligosaccharide released from the acceptor lipid (i.e., before reaction) and labeled with NaB^3H_4. As indicated earlier, this oligosaccharide was a $Man_5(GlcNAc)_2$. On the other hand, the reaction product that had become labeled with [^{14}C]-

Table II: Donor Substrate Specificity

substrate	amount of radioactivity used (cpm)	radioactivity incorporated in lipid-linked oligosaccharide (10:10:3 fraction) with active enzyme (cpm)	with denatured enzyme (cpm)
Dol-P-[^{14}C]Man	12000	2477	355
Dol-P-[^{3}H]Glc	12500	291	167
Dol-PP-[^{14}C]$GlcNAc_2$	25000	185	213
Dol-PP-[^{14}C]GlcNAc	20000	163	129
UDP-[^{3}H]GlcNAc	50000	193	113
UDP-[^{3}H]Glc	50000	118	187
GDP-[^{14}C]Man	50000	98	210

mannose migrated more rapidly on the Bio-Gel column, suggesting that it had one additional hexose residue. Furthermore, as shown by the lowest profile, the product was susceptible to digestion by Endo H, indicating that it had an α1,3-mannose on the 6-linked mannose branch. On the other hand, the acceptor lipid is not susceptible to digestion by Endo H. This [^{14}C]mannose is released from this oligosaccharide by treatment with jack bean α-mannosidase (data not shown). Thus, the mannose that is added by this partially purified mannosyltransferase must be added to the 6-linked mannose in an α1,3-linkage. The $Man_6(GlcNAc)_2$ was also reduced with unlabeled $NaBH_4$ and identified by HPLC. The product migrated with the expected Man_6-GlcNAc-GlcNAc-ol, and the Endo H treated material corresponded to Man_6-GlcNAc-ol (after $NaBH_4$ reduction).

Properties of the Partially Purified Mannosyltransferase. The incorporation of mannose from dolichyl-P-[^{14}C]mannose into the lipid-linked oligosaccharide [i.e., $Man_6(GlcNAc)_2$-PP-dolichol] with the partially purified enzyme was linear with time of incubation for up to 10 or 15 min and with the amount of enzyme added to the incubations, up to 0.05 mg of protein (data not shown). The partially purified mannosyltransferase showed an optimum pH of 6.5 with activity falling off sharply above and below this pH value (data not shown). The effect of concentration of the substrates, dolichyl-P-mannose and $Man_5(GlcNAc)_2$-PP-dolichol, on the reaction was also examined. The apparent K_m values for the donor substrate, dolichyl-P-[^{14}C]mannose, and the acceptor lipid, $Man_5(GlcNAc)_2$-PP-dolichol, were found to be 1.8×10^{-6} M and 1.6×10^{-6} M, respectively (data not shown).

The specificity of the purified mannosyltransferase was examined with a variety of radiolabeled nucleoside diphosphate sugars and lipid-linked monosaccharides as indicated in Table II. It can be seen from the data presented in this table that only dolichyl-P-[^{14}C]mannose was active as a glycosyl donor and no transfer of radioactivity into lipid-linked oligosaccharide occurred when this donor was replaced with other lipid-linked saccharides such as dolichyl-P-[^{3}H]glucose, dolichyl-PP-[^{14}C]GlcNAc, or dolichyl-PP-[^{14}C]GlcNAc-GlcNAc. In addition, various nucleoside diphosphate sugars, such as GDP-[^{14}C]mannose, UDP-[^{3}H]glucose, and UDP-[^{14}C]-GlcNAc could not serve as glycosyl donors. These studies establish that the partially purified mannosyltransferase is quite specific for dolichyl-P-mannose as the mannosyl donor and is not contaminated with other glycosyltransferases that add glucose or GlcNAc to lipid-linked oligosaccharides.

As indicated earlier, the mannosyltransferase required a metal ion for activity and was most active with Ca^{2+}. Figure 7 shows that the partially purified mannosyltransferase had an almost absolute requirement for Ca^{2+} and this could not be replaced by either Mg^{2+} or Mn^{2+}. The curve shows that the incorporation of mannose into lipid-linked oligosaccharide

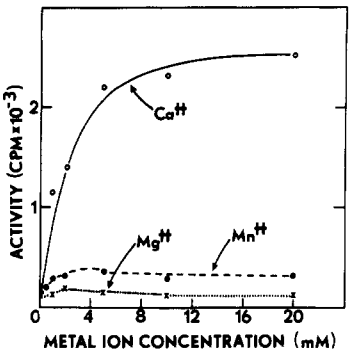

FIGURE 7: Effect of metal ion concentration on the activity of the mannosyltransferase. Reaction mixtures were as described in the text except that the concentration of metal ion, or the metal ion used, was added as indicated in the figure. Control incubations without metal ion contained 3–5 mM EDTA. The activity of the mannosyltransferase was determined as described in the text.

increased with increasing concentration of Ca^{2+} with optimum activity occurring at about 8–10 mM.

Discussion

The present study is the first report on the purification of any of the glycosyltransferases that participate in later stages of the assembly of the lipid-linked saccharides. The first seven sugars of the lipid-linked oligosaccharides, i.e., two GlcNAc and five mannose, are added to the lipid from their nucleotide diphosphate sugar donor, whereas the last four mannose residues and the three glucose units are added from the lipid donors, dolichyl-P-mannose and dolichyl-P-glucose (Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985; Elbein, 1987).

A number of the enzymes including the mannosyltransferases that participate in the early stages of assembly have been partially purified. For example, mannosyltransferase II that catalyzes the addition of the α1,3-linked mannose from GDP-mannose was highly purified from rat liver microsomes (Jensen & Schutzbach, 1981), while an α1,2-mannosyltransferase which also uses GDP-mannose as the mannosyl donor was also purified from that tissue (Schutzbach et al., 1980). However, in spite of these timely and important studies, virtually nothing is known about the regulation of this pathway.

The mannosyltransferase that adds the sixth mannose to the lipid-linked oligosaccharide appears to be a key enzyme in the pathway of biosynthesis of N-linked oligosaccharides for a number of reasons. In the first place, this enyme is the first of the mannosyltransferases that uses dolichyl-P-mannose as the mannosyl donor (Chapman et al., 1980; Chambers et al., 1977). On the other hand, the mannosyltransferases that are involved in the assembly of the $Man_5(GlcNAc)_2$-PP-dolichol use GDP-mannose as the mannosyl donor, suggesting that the oligosaccharide portion of the lipid-linked oligosaccharide may be directed toward the cytoplasm until the addition of the sixth mannose (Hirschberg & Snider, 1987). A second reason that may make this enzyme of special significance revolves around the observations that when cultured cells are perturbed in any one of a number of ways, they use an alternate pathway of biosynthesis of N-linked oligosaccharides. Thus, when various cells are starved for glucose (Turco, 1980) or when they are incubated with energy inhibitors such as CCCP (Datema & Schwartz, 1981), these cells accumulate $Man_5(GlcNAc)_2$-PP-dolichol, probably add three glucose residues to this lipid, and transfer the $Glc_3Man_5(GlcNAc)_2$ to protein. These results suggest that the mannosyltransferase that adds the sixth mannose is very sensitive to these perturbations or is somehow more intimately involved in this shift to the "alternate" pathway. A third reason for the attention to this step in the pathway is that this may be the point where there is a shift in the topography of the lipid-linked oligosaccharide from its orientation toward the cytoplasm to an orientation into the lumen of the ER. Thus, studies by Snider and colleagues (Snider & Robbins, 1982; Snider & Rogers, 1984) have indicated that a change in orientation of the lipid-linked oligosaccharide from a cytoplasmic to a luminal location occurs at the $Man_5(GlcNAc)_2$-PP-dolichol stage.

Although we have been able to purify this mannosyltransferase to a considerable extent, we have not been able to detect any regulation of this enzyme by substrates, products, or other reaction components (phospholipids, nucleotides, etc.). Thus, if this is a point of regulation in the pathway, it may involve other as yet unknown factors of control. It may be, for example, that this mannosyltransferase is inhibited by certain lipid-linked oligosaccharides such as $Glc_3Man_9(GlcNAc)_2$-PP-dolichol or $Glc_3Man_5(GlcNAc)_2$-PP-dolichol if such intermediates accumulate within the cell. However, these lipid-linked oligosaccharides are difficult to obtain in sufficient amounts and in a pure enough state to test as inhibitors. Attempts are now underway to isolate enough of these glycolipids to use for these purposes.

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Isolation and Partial Structural Characterization of an Equine Fibrinogen CNBr Fragment That Exhibits Immunologic Cross-Reactivity with an Aα-Chain Cross-Linking Region of Human Fibrinogen†

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ABSTRACT: Immunochemical studies of equine fibrinogen were conducted to characterize the structural basis for the immunologic cross-reactivity observed between human and equine Aα chains when employing an antiserum to the 26K, human cyanogen bromide (CNBr) fragment, Aα 241–476 (CNBr VIII). A 38K, equine CNBr fragment that reacts with this antiserum was isolated from CNBr-digested equine fibrinogen by Sephadex G-100 gel filtration. It was further purified by sequential hydrophobic chromatography on phenyl-Sepharose CL-4B, followed by reversed-phased (C-8) high-performance liquid chromatography (HPLC). NH_2-Terminal analysis of the purified fragment, designated EqAαCNBr, identified one major sequence whose first three residues, E-L-E, were identical with those of human CNBr VIII. Tryptic and staphylococcal protease digests of the equine fragment were resolved by reversed-phase HPLC (C-4, C-18), and the separated components were characterized by amino acid analysis and automated Edman degradation. A total of 34 tryptic and 20 staph protease peptides yielded sequence information that permitted the alignment of 271 equine residues with residues Aα 241–517 from the COOH-terminal two-thirds of the human Aα chain so that 63% of the possible matches were identical. Other features of interest included (1) an amino acid substitution in which the methionine residue at Aα 476 in the human Aα chain was replaced by a valine residue, thus accounting, in part, for the larger EqAαCNBr fragment obtained from the equine molecule, and (2) a region of striking homology in which 36 successive residues, corresponding to Aα 428–464 in the human Aα chain, were identical in both species. These findings, together with available structural data for the COOH-terminal portion of the rat and bovine Aα chains, indicate that the region corresponding to (human) Aα 240–517 represents a conserved portion of the fibrinogen molecule. This may, in turn, explain the difficulties encountered when trying to raise monoclonal antibodies to cross-linking regions that are contained within the COOH-terminal two-thirds of the human Aα chain.

The COOH-terminal portion of the Aα chain of human fibrinogen contains several structural domains that function to maintain hemostasis by mediating the interaction between fibrin(ogen) and a variety of plasma proteins and cellular components. Included among these domains are factor $XIII_a$ cross-linking regions that foster the covalent interaction between α chains of neighboring fibrin molecules (Doolittle et al., 1977; Fretto & McKee, 1978; Sobel et al., 1983), between α chains and α_2-antiplasmin (Sakata & Aoki, 1980; Tamaki & Aoki, 1982), and between α chains and fibronectin (Mosher, 1975; Sobel et al., 1983). Plasmin-sensitive cleavage sites are also clustered within the COOH-terminal two-thirds of the Aα chain (Pizzo et al., 1970; Takagi & Doolittle, 1975), and an RGDS platelet recognition site has been localized to this region as well (Gartner & Bennett, 1985; Hawiger et al., 1989).

While these various interactions provide a role for the COOH-terminal portion of the Aα chain in maintaining hemostasis, the mechanisms by which this is achieved, in most cases, remain poorly defined. As one approach toward elu-

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