Biosynthesis of Man-β-GlcNAc-GlcNAc-Pyrophosphoryl-Polyprenol by a Solubilized Enzyme from Aorta

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Summary

The particulate enzyme fraction from pig aorta was treated with Triton X-100 or Nonidet P-40 to yield a soluble enzyme preparation. This solubilized enzyme catalyzed the transfer of mannose from GDP-[^{14}C]mannose, but not from [^{14}C]mannosyl-phosphoryl-polyprenol, to GlcNAc-GlcNAc-pyrophosphoryl-polyprenol to form the trisaccharide-lipid, Man-β-GlcNAc-GlcNAc-pyrophosphoryl-polyprenol. The trisaccharide lipid formed in these reactions was isolated by solvent fractionation and was subjected to mild acid hydrolysis to release the [^{14}C]trisaccharide. Essentially all of the radioactivity was released from this trisaccharide as mannose upon treatment with β-mannosidase while α-mannosidase had no effect.

Introduction

The biosynthesis of the $(Man)_n$-GlcNAc-GlcNAc core oligosaccharide of many glycoproteins appears to be mediated through the participation of polyprenyl-linked saccharide intermediates (1-8). Thus, mannose is transferred, presumably from GDP-mannose, to GlcNAc-GlcNAc-pyrophosphoryl-polyprenol to form Man-β-GlcNAc-GlcNAc-pyrophosphoryl-polyprenol (9,10). This trisaccharide-lipid then undergoes additional mannosylation (and perhaps glucosylation) reactions to give an oligosaccharide-lipid which is the precursor to the glycoprotein (9,10). In this report, we describe the solubilization of the mannosyl-transferase which catalyzes the transfer of mannose, from GDP-mannose, to the GlcNAc-GlcNAc-lipid to form Man-β-GlcNAc-GlcNAc-lipid. This soluble enzyme required the addition of GlcNAc-GlcNAc-lipid as an acceptor of mannose. Mannosyl-phosphoryl-polyprenol was inactive as a mannosyl donor. The trisaccharide was isolated and partially characterized as Man-β-GlcNAc-GlcNAc.

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Materials and Methods

The GlcNAc-GlcNAc-pyrophosphoryl-polyprenol was synthesized using a particulate extract from pig liver. Two kg of pig liver were ground in a meat grinder and were then briefly blended in a Waring blender in five volumes of 0.05 M Tris buffer, pH 7.5 containing 2 mM β-mercaptoethanol. The homogenate was filtered through cheesecloth and the supernatant was centrifuged at 30,000 x g for 30 min. The pellet was resuspended in 4 l of 0.05 M Tris buffer and was incubated with UDP-[^{3}H]GlcNAc (5 x 10^6 cpm, 1 μM) in the presence of 3 mM $MnCl_2$ for 15 min. Then a second addition of unlabeled UDP-GlcNAc (20 μM) was made after 15 minutes of incubation. The reaction was stopped by the addition of chloroform:methanol (1:1) and the $(GlcNAc)_2$-lipid was isolated as described previously (6,11). The GlcNAc-lipids were then purified by chromatography on DEAE-cellulose (see Figure 1).

The particulate enzyme from pig aorta was prepared from the intima-media layer as previously described (6,11). The enzyme was solubilized by treatment of the particulate enzyme with Nonidet P-40 as follows: ten ml of particulate enzyme was stirred for about 15 minutes at 5° in 0.5% Nonidet P-40. The mixture was then centrifuged at 100,000 x g for 60 minutes and the supernatant liquid was used as the enzyme source. Assays for the synthesis of the trisaccharide-lipid were prepared in the following way. Variable amounts of the GlcNAc-GlcNAc-pyrophosphoryl-polyprenol were added to incubation tubes and the solvent was removed under a stream of nitrogen. The lipid was then suspended 0.35 ml of solubilized enzyme (0.7 mg protein) and other reaction components were added as follows to give a final volume of 0.4 ml: Tris buffer, pH 7.5, 20 μmoles; $MgCl_2$, 3 μmoles and GDP-[^{14}C]mannose (90,000 cpm 0.4 M). Following an incubation of 15-30 minutes at 37°, the reaction was stopped by the addition of 2 ml of chloroform:methanol (1:1) and 0.5 ml of water and lipids were isolated as described previously (6,11).

Oligosaccharides were released from lipid-linked oligosaccharides by mild acid hydrolysis in 0.5 N HCl at 100° for 15 minutes. Oligosaccharides were chromatographed on Whatman #1 paper in the following solvent systems: A) n-butanol:pyridine:H_2O (40:30:40), B) n-butanol:pyridine:0.1 N HCl (5:3:2), C) ethyl acetate:acetic acid:formic acid:H_2O (18:3:1:4). Sugar standards were detected with the periodate-permanganate spray (12). Lipids were chromatographed on thin layer plates (0.25 mm, Analtech) in the following solvent systems: D) chloroform:methanol:H_2O (65:25:4); E) chloroform:methanol:ammonium hydroxide (25:25:4); F) chloroform:methanol:H_2O (60:25:4); and G) chloroform:methanol:acetic acid:H_2O (50:25:7:3). β-Mannosidase was purified to homogeneity from Aspergillus niger as described (13) and α-mannosidase was partially purified from jack bean meal (14). GDP-[^{14}C]mannose was purchased from New England Nuclear Co. (278 Ci/ mole).

Results and Discussion

The GlcNAc-disaccharide lipid was synthesized with a particulate extract of pig liver. This lipid was purified on columns of DEAE-cellulose as shown in Figure 1. Aliquots of every other fraction were assayed for radioactivity in order to locate the ^{3}H-labeled-GlcNAc-GlcNAc-lipid. Another aliquot of every other fraction was tested for its ability to stimulate the incorporation of mannose from GDP-[^{14}C]mannose into the trisaccharide-lipid using the soluble enzyme from pig aorta. As shown in the Figure, the ^{3}H-radioactivity, representing

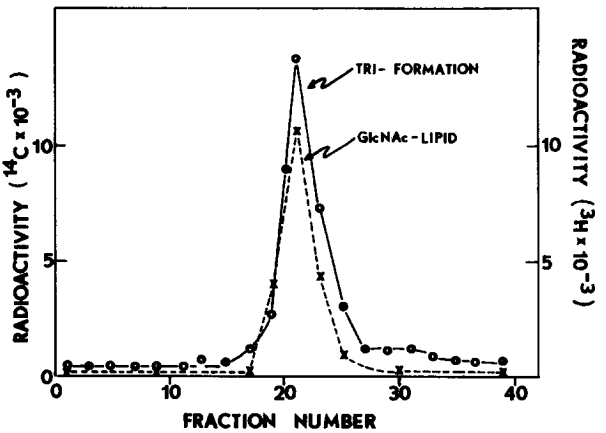

Figure 1. DEAE-cellulose chromatography on biosynthesized ^{3}H-GlcNAc-GlcNAc-pyrophorphoryl-polyprenol. Lipids were eluted with a gradient of 0-0.25 M ammonium acetate. Aliquots were removed for determination of ^{3}H while another aliquot was tested as an acceptor of mannose using GDP-[^{14}C]mannose and the solubilized enzyme from pig aorta. Trisaccharide-lipid formation was determined by chromatographing the ^{14}C-products released by mild acid hydrolysis of the lipids. x-x, [^{14}C]trisaccharide isolated by paper chromatography; o-o, total radioactivity present as ^{3}H per fraction.

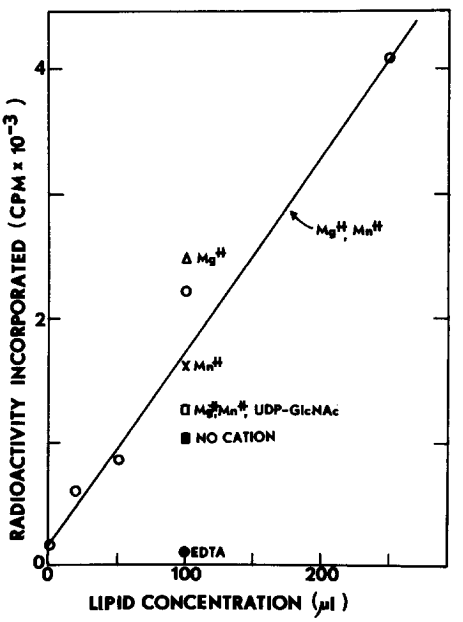

Figure 2. Formation of trisaccharide-lipid as a function of concentration of $(GlcNAc)_2$-lipid. Incubations were as described in the text with GDP-[^{14}C]mannose and solubilized enzyme. Trisaccharide was released from lipid by mild acid and was isolated by paper chromatography.

the $(GlcNAc)_2$-lipid emerged in the same area of the column as the stimulatory activity, suggesting that the $(GlcNAc)_2$-lipid is the acceptor of mannose. Also shown in this figure is the fact that in the absence of acceptor lipid, the soluble enzyme was not able to catalyze the incorporation of mannose into the trisaccharide-lipid. Active fractions from the DEAE-cellulose column (18-26) were pooled and the lipid was extracted into chloroform to remove the ammonium acetate. Mild acid hydrolysis of an aliquot of this lipid revealed that most of the ^3H migrated with N,N'-diacetylchitobiose while a small amount of ^3H migrated with GlcNAc. Thus, the bulk of the ^3H-labeled material in this peak is apparently GlcNAc-GlcNAc-pyrophosphoryl-polyprenol (11).

Figure 2 shows the effect of concentration of GlcNAc-GlcNAc-pyrophosphoryl-polyprenol on the formation of the trisaccharide-lipid using the soluble enzyme from pig aorta. The reaction was proportional to the amount of lipid added from 10-250 µl. It should be pointed out that it was not possible to determine the actual amount of $(GlcNAc)_2$-lipid added to these incubations, since the concentrations of GlcNAc were too low for colorimetric determination. Although saturation was not reached in this experiment, in other cases with different lipid preparations, the reaction did level off at higher lipid concentrations. While the reaction did not show an absolute requirement for a divalent cation, it was inhibited by the addition of EDTA, suggesting that a metal ion is necessary for activity. As shown in the Figure, Mg^{++} was the best cation and showed optimal activity at a concentration of 2 mM. The addition of unlabeled UDP-GlcNAc appeared to have a slight inhibitory effect. The trisaccharide lipid was examined by thin layer chromatography and found to migrate with the following mobilities: 0.25 in solvent system D; 0.01 in solvent E; 0.31 in solvent G; and 0.2 in solvent system F. In each case the trisaccharide lipid migrated much slower than mannosyl-phosphoryl-polyprenol and slightly slower but well separated from $(GlcNAc)_2$ pyrophosphoryl-polyprenol.

In order to determine the nature of the mannosyl donor in these reactions, incubations were prepared which contained GlcNAc-GlcNAc-lipid, Mg^{++} and soluble

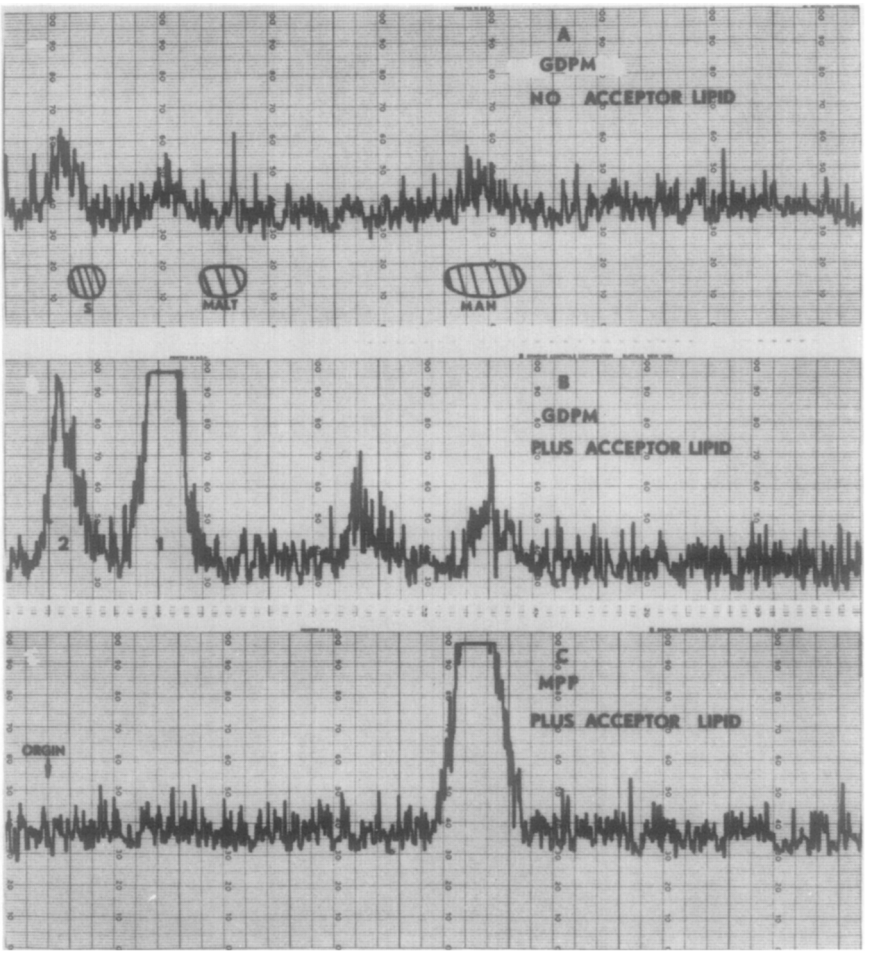

Figure 3. Paper chromatography of oligosaccharides formed from GDP-[^{14}C]mannose or [^{14}C]mannosyl-phosphoryl-polyprenol. Scan A contained GDP-[^{14}C]mannose but no $(GlcNAc)_2$-lipid. Scan B contained GDP-[^{14}C]mannose plus $(GlcNAc)_2$-lipid. Scan C contained [^{14}C]mannosyl-phosphoryl-polyprenol plus $(GlcNAc)_2$-lipid.

enzyme and either GDP-[^{14}C]mannose or [^{14}C]mannosyl-phosphoryl-polyprenol as the mannosyl donor. Following incubation and isolation of the lipids, the [^{14}C]glycolipids were subjected to mild acid hydrolysis and the aqueous phases were chromatographed on paper in solvent A as shown in Figure 3. It can be seen in tracing B that in the presence of GDP-[^{14}C]mannose and acceptor lipid (GlcNAc-disaccharide lipid), a radioactive peak was observed in the trisaccharide area of the paper (#1 in this tracing). A slower moving radioactive peak (#2) was also observed

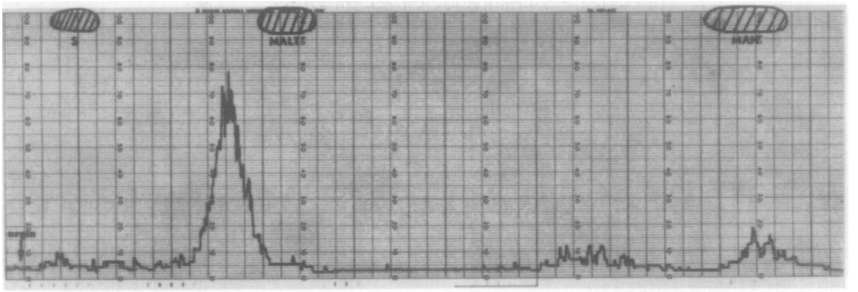

Figure 4. Paper chromatography of Man-$(GlcNAc)_3$ trisaccharide. The trisaccharide was isolated as described in the Text and chromatographed on Whatman #1 paper in solvent A. The radioactivity was located by radiochromatogram scanning. Standards are stachyose (S), maltose (MALT), and mannose (MAN).

which is probably a tetrasaccharide or a pentasaccharide but this has not been further identified at this time. In the absence of acceptor lipid (tracing A), only a small amount of radioactivity was found in the trisaccharide, suggesting again that the $(GlcNAc)_2$-lipid is probably an acceptor of mannose. Further, as indicated in tracing C, no radioactivity was seen in the trisaccharide when mannosyl-phosphoryl-polyprenol was used as the mannosyl donor. Thus, GDP-mannose appears to be the direct donor for the addition of the first mannose residue in the oligosaccharides of these lipids. It has been postulated that this first mannose residue comes from GDP-mannose since this transfer involves an inversion of configuration (1) and some workers have presented evidence for this (9).

The trisaccharide, isolated by mild acid hydrolysis of the trisaccharide-lipid, was purified by paper chromatography and gel filtration on Sephadex G-15. The radioactive trisaccharide emerged from the G-15 columns just after a stachyose standard. Paper chromatography of this material in solvent A, as shown in Figure 4, revealed a single radioactive peak migrating slightly slower than maltose. This material also gave a single radioactive peak, which migrated like a trisaccharide, in solvents B & C. The trisaccharide was treated with α- and β-mannosidase to determine the anomeric configuration

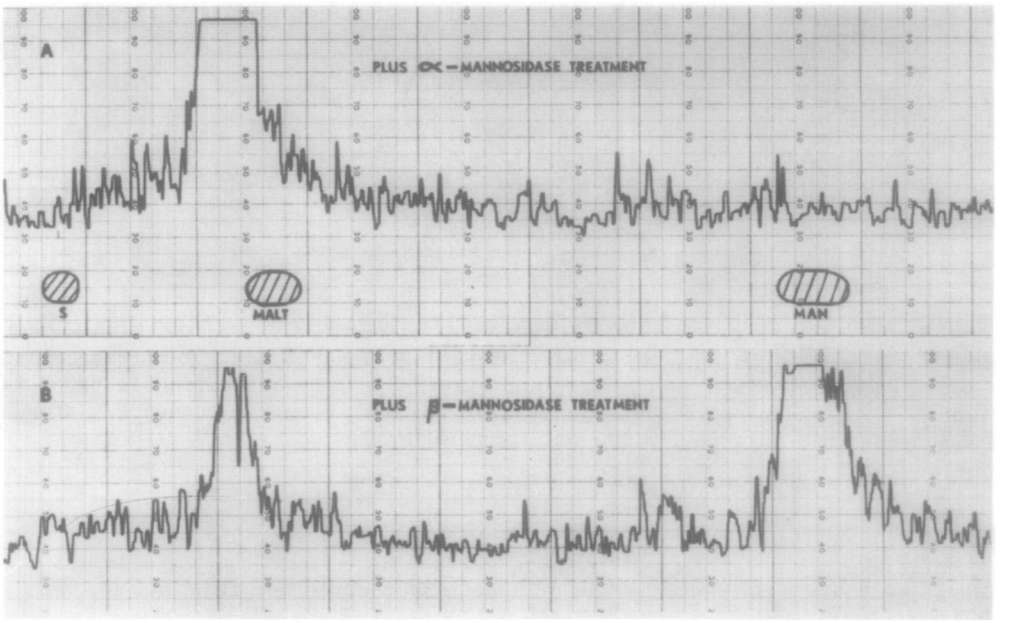

Figure 5. Identification of [^{14}C] products released by α or β-mannosidase digestion of [^{14}C]trisaccharide. Trisaccharide was incubated in 0.01 M acetate buffer, pH 5.0 for 15 hours with either 1 mg of α-mannosidase (upper scan) or 1 mg of β-mannosidase (lower scan). Products were chromatographed in solvent c. Standards are stachyose (S), maltose (MALT) and mannose (MAN).

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of the mannose. As shown in Figure 5, most of the radioactivity was released by incubation with β-mannosidase but α-mannosidase had no effect on this trisaccharide. A second treatment of the remaining trisaccharide with β-mannosidase released the rest of the ^{14}C as mannose. The trisaccharide was reduced with ^{3}H-$NaBH_4$ and then subjected to periodate oxidation. Mild acid hydrolysis gave a ^{3}H-disaccharide which migrated like the periodate oxidized product from ^{3}H-chitotriitol. Thus, the trisaccharide found in these reactions appears to be Man-β-GlcNAc-GlcNAc.

The results described in this paper show that a solubilized mannosyl transferase obtained from pig aorta catalyzes the transfer of mannose from GDP-[^{14}C]mannose to GlcNAc-GlcNAc-pyrophosphoryl-polyprenol to form the Man-β-GlcNAc-GlcNAc-pyrophosphoryl-polyprenol. The solubilized enzyme required the addition of divalent cations. Levy et al. (9) first showed the synthesis

of trisaccharide lipid using a microsomal preparation of oviduct or liver tissue. The addition of $(GlcNAc)_2$-lipid to these tissues along with GDP-mannose was necessary for trisaccharide formation. Wedgwood et al. (10) found that chemically synthesized GlcNAc-GlcNAc-pyrophosphoryl-dolichol would serve as an acceptor of mannose with a membrane fraction from lymphocytes. In those experiments, mannosyl-phosphoryl-polyprenol was inactive as a mannosyl donor, although apparently the oligosaccharides formed were larger than trisaccharides. Recently, Chen and Lennarz have shown the synthesis of this trisaccharide-lipid upon incubation of oviduct membranes with GDP-mannose and GlcNAc-GlcNAc-pyrophosphoryl-dolichol (17). These workers showed that the trisaccharide-lipid could participate in the glycosylation of protein when incubated with these membrane fractions. The finding that the mannosyl transferases can be solubilized by treatment of the aorta particulate enzyme with Nonidet P-40 should make it possible to decipher the individual steps in the synthesis of the oligosaccharide chain. Previous studies from this laboratory have shown that other mannosyl transferases, which add more distal mannose residues, are also solubilized by this detergent treatment (15). In all of these cases, the solubilized enzymes require the addition of exogenous lipid in order to transfer mannose to form the oligosaccharide-lipids. Presumably, this exogenous-lipid serves as an acceptor of mannose residues. We have previously described the isolation of oligosaccharide-lipids from liver which serve as mannose acceptors with particulate and solubilized enzymes from pig aorta (7).

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