Purification and Properties of UDP-GlcNAc:Dolichyl-Pyrophosphoryl-GlcNAc GlcNAc Transferase from Mung Bean Seedling

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Received for publication January 27, 1986

ABSTRACT
The N-acetylglucosamine (GlcNAc) transferase that catalyzes the formation of dolichyl-pyrophosphoryl-GlcNAc-GlcNAc from UDP-GlcNAc and dolichyl-pyrophosphoryl-GlcNAc was solubilized from the microsomal enzyme fraction of mung beans with 1.5% Triton X-100, and was purified 140-fold on columns of DE-52 and hydroxylapatite. The partially purified enzyme preparation was quite stable when stored in 20% glycerol and 0.5 millimolar dithiothreitol, and was free of GlcNAc-1-P transferase and mannosyl transferases. The GlcNAc transferase had a sharp pH optimum of 7.4 to 7.6 and the $K_m$ for dolichyl-pyrophosphoryl-GlcNAc was 2.2 micromolar and that for UDP-GlcNAc, 0.25 micromolar. The enzyme showed a strong requirement for the detergent Triton X-100 and was stimulated somewhat by the divalent cation Mg$.^2$ Uridine nucleotides, especially UDP and UDP-glucose inhibited the enzyme as did the antibiotic, diumycin. However, a variety of other antibiotics including tunicamycin were without effect. The product of the reaction was characterized as dolichyl-pyrophosphoryl-GlcNAc-GlcNAc.

The biosynthesis of the oligosaccharide portion of the N-linked glycoproteins involves the formation of lipid-linked oligosaccharides and transfer of oligosaccharide to protein (7, 18, 28). The formation of the lipid-linked oligosaccharides requires a number of membrane-bound glycosyl transferases that catalyze the addition of GlcNAc, mannose, and glucose to a lipid carrier with the ultimate formation of the tetradecasaccharide-lipid, Glc$_3$Man$_6$(GlcNAc)$_2$-pyrophosphoryl-dolichol (7, 20, 29). Many studies have been done on the lipid-intermediate pathway in animal cells, and several of the glycosyl transferases have been solubilized and partially purified. The Glc$_3$Man$_6$(GlcNAc)$_2$-pyrophosphoryl-dolichol has also been isolated and/or synthesized in several different plant systems (15, 22, 27). However, considerably less information is available on the individual reactions in the lipid-linked saccharide pathway in plants. Previously, we showed that a particulate enzyme fraction from mung bean seedlings incorporated mannose from GDP-mannose into a number of lipid-linked oligosaccharides (16). The oligosaccharides were partially characterized as Man$_5$(GlcNAc)$_2$-PP-lipid, Man$_4$(GlcNAc)$_2$-PP-lipid, Man$_3$(GlcNAc)$_2$-PP-lipid, Man$_2$(GlcNAc)$_2$-PP-lipid, Man$_1$(GlcNAc)$_2$-PP-lipid, and Man$_0$(GlcNAc)$_2$-PP-lipid. Recently, the mannosyl transferases from suspension-cultured soybean cells were solubilized (17). The solubilized enzyme preparation synthesized Man$_n$(GlcNAc)$_2$-lipid, Man$_m$(GlcNAc)$_2$-lipid, Man$_a$(GlcNAc)$_2$-lipid, and Man$_b$(GlcNAc)$_2$-lipid when incubated with lipid acceptors, dolichyl-phosphate and GDP-[$^1^C$]mannose. However, when the incubations were done in the presence of EDTA and amphomycin, both of which inhibit the formation of dolichyl-phosphoryl-mannose, only the Man$_a$(GlcNAc)$_2$-lipid and the Man$_b$(GlcNAc)$_2$-lipid were formed (17). That study suggested that the first five mannose residues in the lipid-linked oligosaccharides come directly from GDP-mannose, whereas the next four mannose units come from dolichyl-phosphoryl-mannose. Similar results were previously reported in animal cells (18, 20). The individual mannosyl transferases have not been purified from either plant or animal systems so that their properties are not known. However, in order to understand the lipid-linked saccharide pathway in detail, it will be necessary to purify each of the glycosyl transferases and determine their requirements for glycosyl donor and glycosyl acceptor. Such studies will also indicate control points in the pathway, if they exist. Thus, we have initiated an attempt to purify as many of the individual glycosyl transferases as possible. In this report, we describe the partial purification of the GlcNAc transferase that adds GlcNAc to GlcNAc-pyrophosphoryl-dolichol to form GlcNAc-GlcNAc-pyrophosphoryl-dolichol. Various properties of the partially-purified enzyme are described.

MATERIALS AND METHODS

Materials. GDP-[$^1^C$]mannose (269 mCi/mm) and UDP-[6-3$^H$]GlcNAc (20 Ci/mmol) were purchased from New England Nuclear Co. Dolichyl-phosphate, unlabeled sugar nucleotides, bacitracin, and showdomycin were obtained from Sigma Chemical Co. Tunicamycin and polyvinylpyrrolidone were from Calbiochem, and hydroxyapatite and Biogel P-4 (200–400 mesh) were from Biorad Co. Amphomycin was a generous gift from Mr. William Minor, Bristol Labs. All other chemicals were from reliable commercial sources and were of the best grade available.

Preparation of Membrane Fraction. Mung beans were soaked in tap water overnight and spread on moist cotton for germination in the dark at room temperature for 2 or 3 d. The sprouts were removed and kept in ice. About 400 g of sprouts were blended for 20 s in 200 ml of 50 mm Tris-HCl buffer (pH 7.5) containing 8% sucrose, 0.5% polyvinylpyrrolidone, 1 mm EDTA, 1 mm MgCl$_2$, and 0.5 mm DDT. The homogenate was strained through eight layers of cheesecloth and centrifuged at 3,000g to remove large particles and whole cells. The resulting supernatant was then centrifuged at 105,000g for 45 min to obtain the membrane pellet. All preparations were done at 0 to 4°C unless otherwise indicated.

Solubilization of GlcNAc Transferase. The membrane fraction
obtained above was suspended to 50 mM Tris-HCl buffer (pH 7.4) containing 10% glycerol and 0.5 mM DTT. The suspension was treated with the nonionic detergent, Triton X-100, at a final concentration of 1.5%. The suspension was gently homogenized over a 20 min period while being kept in ice. The suspension was then centrifuged at 105,000g for 60 min and the supernatant liquid was removed and used as the source of enzyme.

Preparation of Dolichyl-Phosphoryl-GlcNAc. The dolichyl-phosphoryl-GlcNAc that was used as the GlcNAc acceptor was prepared from pig liver, by a modification of a previously described procedure (12). Pig liver (about 2 kg) was blended in 2 L of distilled H₂O and the suspension was made 1:1:1 (CHCl₃:CH₃OH:H₂O) by the addition of CHCl₃ and CH₃OH. The extraction was mixed thoroughly and the phases were allowed to separate. The lower, CHCl₃ phase was removed and saved, and more CHCl₃ (3 L) was added to the mixture. After thorough mixing, the CHCl₃ layer was removed and pooled with the previous CHCl₃ layer. The rest of the suspension was filtered and the residue was resuspended in 5 L of CHCl₃:CH₃OH:H₂O (10:10:3). After standing overnight, the extraction was filtered and the 10:10:3 solution was saved. The residue was reextracted with another 5 L of 10:10:3. The 10:10:3 extracts were pooled, concentrated under vacuo at 35°C, dissolved in 1 L of CHCl₃:CH₃OH:H₂O (10:10:3) containing 0.1 N NaOH and heated at 37°C for 30 min to saponify lipids.

After saponification, the mixture was adjusted to 1:1:1 of CHCl₃:CH₃OH:H₂O by the addition of water, and the lower phase and interface were removed and washed with 50% CH₂OH. The CHCl₃ layer was then removed, concentrated to dryness, and redissolved in 10:10:3. This fraction, containing the dolichyl-phosphoryl-GlcNAc, was applied to a DEAE-cellulose (acetate) column that had previously been equilibrated with 10:10:3. The column was washed with 10:10:3 and the lipid-linked saccharides were eluted with 0.2 M ammonium formate in the same solvent. The ammonium formate eluate was adjusted to 1:1:1 by the addition of water and mixed vigorously. The phases were separated and the lower CHCl₃ phase and interface were again saponified, concentrated and redissolved in 10:10:3. To this 10:10:3 solution, a small amount of radioactive (8000 cpm) dolichyl-phosphoryl-GlcNAc as prepared previously (19), was added as a marker. This mixture was applied to a DEAE cellulose column as described above but in this case the column was eluted with a linear gradient of 0 to 0.2 M ammonium formate. The dolichyl-phosphoryl-GlcNAc emerged at about 0.1 M ammonium formate. The lipid was further purified by TLC on silica gel plates in CHCl₃:CH₃OH:NH₄OH:H₂O (70:44:5:5).

Assay of UDP-GlcNAc:Dolichyl-Phosphoryl-GlcNAc-GlcNAc Transferase. The dolichyl-phosphoryl-GlcNAc (1.2 nmole) was dispersed in 1% Triton X-100, 2 mM MgCl₂, 50 mM Tris-HCl buffer (pH 7.4), 0.1 ml BSA (0.5%), and 0.1 ml of enzyme solution in a final volume of 0.4 ml. The reaction was started by the addition of 0.1 μCi of UDP-[3H]GlcNAc, and the mixture was incubated for 15 min at 37°C. The reaction was terminated by the addition of 0.6 ml of H₂O and 2 ml of CHCl₃:CH₃OH (1:1). The mixture was vortexed vigorously and the phases were separated by centrifugation. The lower, CHCl₃, phase was gently removed and saved. The upper phase and interface were reextracted with another 1 ml of CHCl₃. The CHCl₃ layers were pooled and washed with CHCl₃:CH₃OH:H₂O (3:4:8:47). The lower phase, which contained dolichyl-phosphoryl-GlcNAc-GlcNAc, was counted for the determination of radioactivity. When inhibitors or sugar nucleotides were tested, they were preincubated with the GlcNAc transferase for 1 min, before the reactions were started by addition of UDP-GlcNAc.

Chemical Procedures. Protein was determined by the method of Bradford (5). Lipid-linked saccharides were hydrolyzed in 0.02 N HCl in 30% CH₃OH at 100°C for 20 min. After hydrolysis, the solution was washed with CHCl₃ to remove lipids and the aqueous phase was concentrated to dryness and dissolved in a small volume of water. The sugars were then identified by chromatography on a 1.5 × 150 cm column of Biogel P-4. The column was equilibrated and run in 0.5% acetic acid. A variety of standard oligosaccharides were run on the column including Man₉(GlcNAc)ₓ, Man₃(GlcNAc)ₓ, N,N' -diacetylchitobiose, GlcNAc, and mannose.

RESULTS

Solubilization and Purification of GlcNAc Transferase. A number of detergents were tested for their ability to solubilize the GlcNAc transferase from the mung bean microsomal fraction. About 80 to 90% of the enzymic activity was solubilized by 1.5% Triton X-100 or NP-40. However, these detergents were ineffective below 0.5%. Octylglucoside and sodium deoxycholate were found to be less effective than Triton X-100 or NP-40. Thus, we used Triton X-100, at 1.5% for solubilization.

The solubilized enzyme (18 ml) was applied to a DE-52 column that had been equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 20% glycerol, 0.2% Triton X-100, and 0.5 mM dithiothreitol. The column was washed with 30 ml of this buffer and eluted batchwise with 60 ml portions of 50 mM, 100 mM, 250 mM, and 400 mM NaCl in the same buffer. Figure 1 shows the elution profile. The enzyme activity was eluted with 50 mM NaCl between fractions 27 and 53. Fractions 31 to 49 were pooled and dialyzed overnight against 2 mM sodium phosphate buffer (pH 7.2) containing 20% glycerol, 0.2% Triton X-100, and 0.5 mM DTT. This purification step gave a 15-fold increase in specific activity with almost no loss of activity.

The enzyme from the DE-52 columns (38 ml) was applied to a 2.5 × 6 cm column of hydroxylapatite that had been equilibrated with 2 mM sodium phosphate buffer (pH 7.2) containing 20% glycerol, 0.2% Triton X-100, and 0.5 mM DTT. The column was washed with buffer and then eluted with 120 ml of a linear gradient of 0 to 0.2 M sodium phosphate buffer (pH 7.2) containing 20% glycerol, 0.2% Triton X-100, and 0.5 mM DTT. As shown in Figure 2, the enzyme activity was eluted in fractions 41 to 69. Fractions 45 to 67 were pooled, kept in ice, and used throughout these studies.

Using the above procedure, the GlcNAc transferase was puri-
The purification procedure.

The concentration of dolichyl-pyrophosphoryl-GlcNAc was lost after 10% incubation by 20% containing activity rapidly was lost after storage overnight at 0°C.

Properties of the GlcNAc Transferase. The purified GlcNAc transferase catalyzed the transfer of GlcNAc from UDP-GlcNAc to dolichyl-pyrophosphoryl-GlcNAc to form dolichyl-pyrophosphoryl-GlcNAc-GlcNAc. This reaction was linear with time of incubation for at least 12 min, and was also proportional to enzyme concentration up to 8 μg of protein (data not shown). The enzymic activity was inhibited by the addition of EDTA to the incubation mixtures and this inhibition could be overcome by the addition of divalent cations, especially Mg²⁺. In addition, although the enzyme did not show an absolute requirement for divalent cation, its activity was greatly increased by the addition of Mg²⁺. Mn²⁺ also stimulated activity but not nearly as well (data not shown).

The purified enzyme showed a very sharp pH optimum at 7.4 to 7.6 (data not shown). On either side of this pH optimum, activity rapidly declined. The pH profile for the GlcNAc transferase was similar in either Tris buffer or in Hepes buffer. The purified transferase was greatly stimulated by detergent as shown in Figure 3. The enzyme was normally kept in 0.2% Triton X-100, and therefore it was not possible to completely remove all detergent from the reaction mixtures. However, as the amount of detergent was increased to 0.2% and beyond, there was a dramatic increase in activity. In most of the experiments described here, the incubations contained Triton X-100 at a final concentration of 0.25%.

The effect of substrate concentration on the formation of dolichyl-pyrophosphoryl-GlcNAc-GlcNAc was examined with the partially purified GlcNAc transferase. Figure 4 shows that the formation of dolichyl-pyrophosphoryl-GlcNAc-GlcNAc was proportional to the concentration of dolichyl-pyrophosphoryl-GlcNAc up to about 1.5 or 2 μM, and then began to level off at higher concentrations of substrate. When this data was plotted according to the method of Lineweaver and Burk, the Kₘ for dolichyl-pyrophosphoryl-GlcNAc was estimated to be 2.2 μM. The effect of UDP-GlcNAc concentration on the reaction rate was also determined as shown in Figure 5. The increase in product formation was proportional to UDP-GlcNAc concentration. When this data was plotted according to Lineweaver and Burk, the Kₘ for UDP-GlcNAc was estimated to be 0.25 μM. However, it should be pointed out that these experiments were done at less than saturating levels of UDP-GlcNAc, since it was not possible to dilute the radioactive UDP-GlcNAc with unlabeled sugar nucleotide and still retain sufficient activity.

Identification of the Reaction Products. In order to characterize the product formed from UDP-GlcNAc and dolichyl-pyrophosphoryl-GlcNAc, the lipid-linked saccharide product formed by either the solubilized enzyme or the partially purified enzyme was isolated by solvent extraction. The radioactive oligosaccharides were liberated by mild acid hydrolysis and these oligosaccharides were identified by gel filtration on columns of Biogel P-4. Figure 6 shows the profile obtained from this column with the product of the solubilized enzyme. In this case, the major radioactive product corresponded to N,N'-diacetylmuramylpentapeptide indicating that by far the major product was dolichyl-pyrophosphoryl-GlcNAc-GlcNAc. However, a small peak was also seen that corresponded to Man-GlcNAc-GlcNAc suggesting that the solubilized preparation might contain small amounts of GDP-mannose as well as mannosyl transferase.

However, when the product of the hydroxylapatite-purified transferase was examined, the only oligosaccharide corresponded to N,N'-diacetylmuramylpentapeptide, and no larger-sized oligosaccharides were seen (data not shown). The purified enzyme did not show any UDP-GlcNAc-dolichyl-P:GlcNAc-P transferase activity when incubated with UDP-[³H]GlcNAc and dolichyl-P. The enzyme fraction also did not show any activity when incubated with either dolichyl-phosphate and GDP-[³¹C]mannose or dolichyl-pyrophosphoryl-GlcNAc-GlcNAc and GDP-[³¹C]mannose.

The transferase obtained from hydroxylapatite was free of

Table 1. Purification Procedure of GlcNAc Transferase

A unit of activity for GlcNAc-transferase is defined as the amount of enzyme catalyzing the incorporation of 1000 cpm of [³H]GlcNAc into dolichyl-PP(GlcNAc₂) per 15 min.

<table>
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<tr>
<th>Enzyme Preparation*</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Purification</th>
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<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units</td>
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<tr>
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<td>243</td>
<td>4318</td>
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<td>1</td>
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<tr>
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<td>164</td>
<td>4256</td>
<td>26</td>
<td>1.5</td>
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<tr>
<td>3. DEAE enzyme</td>
<td>38</td>
<td>11</td>
<td>4308</td>
<td>386</td>
<td>22</td>
</tr>
<tr>
<td>4. Hydroxyapatite enzyme</td>
<td>46</td>
<td>1.5</td>
<td>3714</td>
<td>2476</td>
<td>140</td>
</tr>
</tbody>
</table>

*In the incubations of particulate and solubilized enzyme preparations, 0.2 mM AMP was added to prevent the degradation of UDP-[³H]GlcNAc by pyrophosphatases. The rest of the enzyme preparations were assayed as described in the text.
Figure 3. Effect of detergent concentration on GlcNAc transferase activity. Incubation mixtures were as described in the text but the detergent concentration (Triton X-100) was varied as shown. These incubations contained 30 ng of the DE-52 enzyme fraction. The formation of dolichyl-pyrophosphoryl-GlcNAc-GlcNAc was determined as described in the text.

Figure 4. Effect of concentration of dolichyl-pyrophosphoryl-GlcNAc on activity of GlcNAc transferase. Incubation mixtures were as described in the text except that various amounts of dolichyl-PP-GlcNAc were added as indicated in the figure. The incubations contained 30 ng of DE-52 enzyme. The formation of dolichyl-PP-GlcNAc-GlcNAc was determined as described in the text. As shown by the inset, the data were plotted by the method of Lineweaver and Burk.

UDP-GlcNAc pyrophosphatase activity as indicated by the fact that UDP-[3H]GlcNAc was stable when incubated alone with this enzyme. On the other hand, the solubilized enzyme fraction did contain pyrophosphatase activity as shown in Figure 7. It can be seen by panel A, that UDP-[3H]GlcNAc was degraded by the solubilized enzyme to GlcNAc-1-P. This degradation was mostly prevented by the addition of unlabeled GDP-mannose, GDP-glucose, UDP-glucose, AMP, or NADH. However, ATP or ADP did not protect the UDP-GlcNAc from degradation. It is not clear why some nucleotides protect and others do not.

Inhibition of GlcNAc Transferase by Uridine Nucleotides or Antibiotics. The effect of various nucleotides and sugar nucleotides on the GlcNAc transferase was studied with the enzyme purified from hydroxylapatite. In the experiment presented in Figure 8, the ability of the enzyme to form dolichyl-pyrophosphoryl-GlcNAc-GlcNAc was examined in the presence of various amounts of unlabeled uridine nucleotides. It can be seen that UDP was the most potent inhibitor causing 50% inhibition at about 50 µM. UDP-glucose and UMP were also inhibitory but less so than UDP, and UTP was the least effective. The inhibition by UDP is not unexpected since it is one of the products of the reaction.

A number of antibiotics have been reported to effect the lipid-linked saccharide pathway in plants, yeast, and animal cells (7). In plants, tunicamycin was shown to inhibit the formation of dolichyl-pyrophosphoryl-GlcNAc (9), while amphotericin blocked the formation of dolichyl-phosphoryl-mannose and dolichyl-pyrophosphoryl-GlcNAc (10). Another antibiotic, showdomycin, was found to inhibit the GlcNAc-1-P transferase that forms dolichyl-pyrophosphoryl-GlcNAc in green algae (24). Finally, bacitracin was found to inhibit GlcNAc incorporation into lipids in plants but the specific site of inhibition was not determined (11).

As shown in Figure 9, we examined the effects of various amounts of these antibiotics and several others, on the ability of the purified enzyme to catalyze the synthesis of dolichyl-pyrophosphoryl-GlcNAc-GlcNAc. It can be seen that diu-

mecin was a fairly effective inhibitor causing 50% inhibition at about 10 µg/incubation, with progressively more inhibition at higher concentrations. Bacitracin was only slightly effective, except at high concentrations and showdomycin and amphotericin...
Fig. 7. Effect of various nucleotides on degradation of UDP-[3H]-GlcNAc by solubilized enzyme. UDP-[3H]GlcNAc was incubated with the solubilized enzyme (1 mg protein) in the absence (profile A) or presence of the indicated nucleotides. Each was added in the amount of 1 μmol/incubation. After incubation, the reaction products were spotted on Whatman No. 3MM paper and chromatographed in ethanol: 1 m ammonium acetate (pH 7.4). Chromatograms were cut into 1 cm strips and counted in the scintillation counter. The arrow shows the position of standard UDP-GlcNAc.

were without effect. Tunicamycin also did not inhibit this GlcNAc transferase (data not shown).

DISCUSSION

The transfer of GlcNAc from UDP-[3H]GlcNAc into lipids has previously been reported using particulate enzyme preparations from cotton fibers (7), developing cotyledons of Phaseolus vulgaris (8), soybean cells (3) pea cotyledons (4), and mung bean cotyledons (6, 13, 21). Two GlcNAc residues are transferred to dolichyl-phosphate in this system; the first GlcNAc is transferred as GlcNAc-I-P from UDP-GlcNAc, while the second is transferred as GlcNAc from this sugar nucleotide (23). As yet, neither of these enzymes has been solubilized or purified from any plant sources. The first enzyme, i.e. the GlcNAc-I-P transferase has been partially purified from pig aorta (14, 19) and other animal sources (1, 25). The second enzyme, i.e. GlcNAc transferase, was solubilized from yeast (26) but it has not been purified or studied from any other source.

In this report, we describe the purification of the UDP-GlcNAc:dolichyl-phosphoryl-GlcNAc-GlcNAc transferase from mung bean seedlings. The 140-fold purified enzyme was free of GlcNAc-I-P transferase and mannans transferases, and catalyzed the formation of a single product, dolichyl-phosphoryl-GlcNAc-GlcNAc. This transferase was stable for at least a month in the frozen state and for 5 or 6 d in ice, as long as the enzyme was stored in 20% glycerol plus 0.5 mM DTT. Thus, the enzyme is fairly stable under these conditions, allowing a number of studies to be done. Our preliminary studies did not give any indication that this enzyme plays a role in regulation of the pathway although the enzyme was inhibited by certain uridine nucleotides. However, it is likely that these inhibitions represent either product inhibition or substrate analogs. Further studies with this purified enzyme should indicate whether other factors inhibit this enzyme. In terms of other types of inhibitors, diumycin was found to be a fairly effective inhibitor of this activity while amphotomycin, showdomycin, and bacitracin were either inactive or marginally active. Diumycin has previously been reported to inhibit GlcNAc incorporation in yeast (2) and Acanthamoeba (30). However, it has not been tested with any purified glycosyl transferases. If it proves to be specific for this enzyme, it could be of considerable use as a selected inhibitor of the pathway.

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