Purification and Characterization of UDP-N-Acetyl-d-glucosamine:dolichol Phosphate N-Acetyl-d-glucosamine-1-phosphate Transferase Involved in the Biosynthesis of Asparagine-linked Glycoproteins in the Mammary Gland*

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The GlcNAc-1-P-transferase that initiates the dolichol cycle for the biosynthesis of asparagine-linked glycoproteins has been purified from the lactating bovine mammary gland. After solubilization from microsomes with 0.25% Nonidet P-40, the enzyme activity was stabilized with 20% glycerol, 20 μg/ml phosphatidylglycerol, 5 μM dolichol phosphate, and 2.5 μM UDP-GlcNAc. The purification protocol involved (NH₄)₂SO₄ precipitation, gel filtration on Sephacryl S-300, DEAE-TSK, and hydroxylapatite chromatography. The purified enzyme was devoid of several readily detectable glycosyltransferases of the dolichol cycle. It showed two bands (A, 50 kDa and B, 46 kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis after either Coomassie Blue or silver staining. Antibodies (anti-A and anti-B) raised against individual bands A and B inhibited the enzyme activity in solubilized microsomes. Each of the partially purified antibodies recognizes both bands A and B on Western blots of the enzyme; with the solubilized microsomes, the antibodies also recognize an additional polypeptide of ≈70 kDa. When radiolabeled microsomes were immunoprecipitated with anti-B and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, again bands of 46, 50, and 70 kDa were observed. The peptide mapping of 50 and 46 kDa bands of the purified enzyme by chemical cleavage with N-chlorosuccinimide gave similar fragmentation patterns.

The results indicate that the 70 kDa band is a precursor form of the enzyme or this polypeptide, representing the native enzyme or its subunit, is proteolyzed to smaller, enzymatically active peptide(s) of 50 and 46 kDa during purification despite the inclusion of several inhibitors against serine-proteases in all buffers used for tissue homogenization and enzyme purification. A number of properties of the purified enzyme, including its specific activation by Man-P-Dol were also characterized.

Asparagine-linked glycoproteins participate in a wide variety of cellular functions and have been implicated in cell-cell recognition and communication phenomena that are fundamental to biological recognition. An understanding of the control of biosynthesis of the carbohydrate moieties of these proteins is extremely important. Even more significantly, a knowledge about the regulation of these conjugated proteins in hormonally responsive tissues is essential for understanding the mechanisms underlying growth and differentiation.

The biosynthesis of asparagine-linked glycoproteins is initiated by the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichol phosphate to form GlcNAc-P-P-dolichol. Subsequent transfer of GlcNAc, Man, and Glc by membrane-bound glycosyltransferases of the dolichol cycle culminates in an ordered assembly of the branched tetradecasaccharide Glc-Man-GlcNAc₆, the major glycosylation precursor in this pathway (1). Based upon the available evidence, a concerted action of up to 16 glycosyltransferases may be postulated for the assembly of this oligosaccharide. Among these, 14 enzymes would participate in the stepwise construction of the tetradecasaccharide; two glycosyltransferases are required for the biosynthesis of Man-P-Dol and Glc-P-Dol, intermediary donors for four of the mannosylation and the three glycosylation steps, respectively, in the cycle.

The reactions of the dolichol cycle are known to occur in the rough endoplasmic reticulum of the cell (1). The glycosyltransferases of the multienzyme sequence comprising the dolichol cycle are poorly defined due to their membrane-bound characteristic, extreme instability, and unavailability of dolichol-linked saccharides for use as enzyme substrates. These difficulties notwithstanding, a number of reports have appeared in which some of the enzymes have been solubilized by sonication (2-4), phospholipase A, treatment (5, 6), or the use of detergents (7-15) and partially purified and characterized.

As the first step in the multistep sequence of oligosaccharide assembly, the formation of GlcNAc-P-Dol in a reaction catalyzed by UDP-GlcNAc: Dol P-GlcNAc-1-phosphotransferase (GlcNAc-1-P-transferase) would appear to be crucial from the standpoint of regulation of the biosynthetic pathway. Among the studies conducted with microsomal and solubilized preparations of different tissues, the enzyme from Acanthamoeba castellani was found to be stable for several months at 4 °C (4). The solubilized enzyme from rat lung was shown to require phosphatidylglycerol for activity; the enzyme was stabilized by dolichol phosphate (5, 6). The GlcNAc-1-P-transferase in microsomal preparations from several tissues was shown to be activated by Man-P-Dol (16-19). Kausahl

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1. The abbreviations used are: GlcNAc, N-acetyl-d-glucosamine; Dol, dolichol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
and Elbein (16) partially purified GlcNAc-1-P-transferase after solubilization of microsomes from pig aorta with Nonidet P-40. During purification, a heat-stable factor was separated from the enzyme. This factor was shown to stimulate the enzyme about 5-fold. The precise nature of this factor is presently unknown.

The mammary gland is characterized by a cyclic series of highly ordered changes in its biochemistry and physiology during development throughout the reproductive life of the mammalian female. The growth and differentiation of this gland are profoundly modulated by mammogenic and lactogenic hormones (20). Thus, this tissue should serve as an excellent model for studying the potential regulation of hormonally modulated gene expression with regard to glycoprotein biosynthesis. An earlier study from our laboratory showed that three key glycosyltransferases, viz., UDP-GlcNAc:Dol-P GlcNAc-1-P, GDP-Man:Dol-P mannosyl-, and UDP-Glc:Dol-P glucosyltransferase undergo differentiation-related activation during ontogeny of the mouse mammary gland; prolactin appears to play a role in this regulation (21). The enzyme GlcNAc-1-P-transferase is a likely target for hormonal regulation of the biosynthesis of asparagine-linked glycoproteins in the mammary gland. The present paper describes the purification and characterization of this enzyme from the bovine mammary microsomes. Polyclonal antibodies raised against the enzyme exhibit its activity in immunoprecipitation assay and specifically recognize the enzyme polypeptides on Western blot.

EXPERIMENTAL PROCEDURES

Materials—UDP-[3H]glucosamine (269 mCi/mmol), UDP-[14C]mannose (269 mCi/mmol), and UDP-N-acetyl-[14C]glucosamine (20 Ci/mmol) were purchased from Du Pont-New England Nuclear. UDP-[3H]galactose (254 mCi/mmol) and carrier-free Na251 were obtained from Amersham Corp. Dolichol phosphate, adenosine 5'-[alpha-(32P) -methylene]triphosphate, diitho-treitol, phosphatidylglycerol, other phospholipids, concanavalin A-Sepharose, and common chemicals were products of Sigma. The endoglycosidases endo-β-N-acetylglucosaminidases H and F were obtained from Miles Laboratories. 2,3-Dimercapt-1-propanol was purchased from Aldrich. Sephacryl S-300 was from Pharmacia LKB Biotechnology Inc. Hydroxyapatite, chemicals used in SDS-PAGE, and protein A-Sepharose were products of Bio-Rad. DEAE-TSK was obtained from R. M. Science (Cherry Hill, NJ). IODO-BEADS were purchased from Pierce. Anti-rabbit IgG-alkaline phosphatase-conjugated antibodies and the color development reagent were purchased from Fromega Biotech (Madison, WI).

Man-P-Dol and Glc-P-Dol were prepared by incubating GDP-Man or UDP-Glc with dolichol phosphate and bovine mammary microsomes and recovering lipids soluble in CHCl3/CH3OH, 2:1 (22). A trace amount of [3H]Man-P-Dol or [14C]Glc-P-Dol was added to the crude individual mixtures of glycolipids to monitor subsequent purification by four repetitions of chromatography on columns of DEAE-cellulose (23). The purified glycolipids showed a single phosphatase-positive spot when examined by TLC in three different solvent systems (23).

Preparation of Solubilized Enzyme—All the buffers used throughout tissue homogenization and purification contained antiproteases α-phenanthroline (1 mM), phenylmethylsulfonyl fluoride (0.5 mM), and ε-amino- caproic acid (1 mM). Unless stated otherwise, all operations were conducted at 2-4°C. The particular enzyme was prepared from the lactating bovine mammary tissue using the procedure previously described (22). The membranes were resuspended in 20 mM Tris-HCl pH 7.6, containing 1 mM EDTA, 10 mM β-mercaptoethanol, 10 mM MgCl2, 10% glycerol, and 0.25% Nonidet P-40 at a final protein concentration of 20 mg/ml. The suspension was stirred for 15 min on a magnetic stirrer and then centrifuged at 200,000 × g for 1 h. The supernatant was collected and additional glycerol (final concentration 20%), dolichol phosphate (added from a 1 mM stock in 0.1% Nonidet P-40; final concentration, 5 μM), and phosphatidyl glycerol (50 μg/ml stock solution) were added back. Boiling at 100°C in the presence of 5 mg/ml proteinase K (final concentration, 20 μg/ml protein), and unlabeled UDP-GlcNAc (final concentration, 2.5 μM) were added. This preparation of enzyme was stable for at least 1 week at 2-4°C with less than 10% loss in activity.

Preparation of Subcellular Fractions—The mammary tissue was fractionated to obtain plasma membrane, Golgi, and rough endoplasmic reticulum essentially as described by Carey and Hirschberg (24) except that all sucrose solutions contained 50 mM Tris-HCl, pH 7.6.

Purification of the Enzyme—300 ml of the solubilized enzyme were brought to 50% ammonium sulfate saturation by adding solid (NH4)2SO4. After standing on ice for 1 h, the precipitate was removed by centrifugation. The precipitate was dissolved in buffer A (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 5 mM β-mercaptoethanol, 5 mM MgCl2, 20 μg/ml phosphatidylglycerol, 2.5 μM UDP-GlcNAc, and 0.1% detergent Nonidet P-40) and subjected to gel filtration on a column of Sephacryl S-300 (6 × 52 cm) eluted with buffer A (Fig. 1A); 5-ml fractions were collected and checked for enzyme activity and protein concentration. The fractions containing the peak of enzyme activity were pooled and applied to a column (1.5 × 50 cm) of DEAE-TSK equilibrated with buffer B (same as buffer A but without the detergent). This column was attached to a Fast Protein Liquid Chromatograph (Pharmacia). The column was washed with 50 ml of buffer B followed by another 150-ml wash with buffer B containing 150 mM KC1 in linear gradient of 0.15-1.0 M KC1 in buffer A. A flow rate of 2.5 ml/min and fraction size of 5 ml were programmed (Fig. 1B). The column fractions were monitored for protein as well as enzyme activity. The peak fractions containing the enzyme activity were pooled and desalted on a column of Bio-Gel P-2 (3 × 55 cm) in buffer A. The desalted enzyme fractions were applied to a column (1.5 × 7 cm) of hydroxyapatite equilibrated in buffer A and attached to the fast protein liquid chromatography system. An elution sequence consisting of a 50-ml wash with buffer A, another 50-ml wash with buffer A containing a linear increase in KC1 concentration to 0.15 M followed by a linear gradient of 0.15-1.0 M KC1 in buffer A with a flow rate of 2.5 ml/min and a fraction size of 5 ml were programmed (Fig. 1C). The peak fractions were collected in polypropylene tubes containing glycerol (final concentration 40%), dolichol phosphate (final concentration, 5 μM), and phosphatidylglycerol (final concentration, 10 μg/ml) to stabilize the enzyme, which is extremely unstable at this stage. Under these conditions, the purified enzyme lost nearly 80% of its activity after 24 h. Unless stated otherwise, all studies were conducted with the purified enzyme after the hydroxyapatite step, as soon as possible.

Assay of Glycosyltransferases and Marker Enzymes—Three enzymes, viz., UDP-GlcNAc:Dol-P GlcNAc-1-P-transferase, GDP-Man:Dol-P mannosyltransferase, and UDP-Glc:Dol-P glucosyltransferase were assayed using a standard reaction mixture that contained 50 mM Tris-HCl, pH 7.6, 10 mM diithiothreitol, 10 mM MgCl2, 0.3 mM adenosine-5'-α-(32P)-methylene)triphosphate, 5 mM 2,3-dimercapt-1-propanol, 5 mM EDTA, 20 μg/ml phosphatidylglycerol, and a 1 mM stock solution in 1.0% Nonidet P-40), up to 20 μg of protein, and the radioactive sugar-nucleotide in a volume of 0.1 ml. The final concentration of detergent in the incubation mixture was 0.05%. For the assay of GlcNAc-1-phospho- and glucosyltransferase, 1.0 μl of the corresponding 3H-labeled sugar-nucleotide was used; for the assay of mannosyltransferase, 0.4 μl of GDP-[3H]mannose was used. After incubation for 10 min at 37°C, reactions were stopped with 2 ml of CHCl3/CH3OH, 2:1, and processed to obtain the chloroform-methanol-soluble products as described (22). Under these conditions, the reaction rates were linear with respect to time for 15 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the incorporation of 1000 cpm of radioactive sugar into product(s) under the conditions of the assay. Galactosyltransferase was determined as described (25). The activity of 5'-nucleotidase was assayed according to Aronson and Touster (26) measuring the inorganic phosphate formed from ATP. 80 μl of ATP (25 μCi/mmol) were added to the reaction mixture for 15 min.

Preparation of Antibodies—Antibodies against bovine mammary GlcNAc-1-P-transferase were raised in New Zealand white female rabbits as follows: 150 μg of purified GlcNAc-1-P-transferase was subjected to preparative SDS-PAGE under reducing conditions. After the gel was sliced into two parts, two polyclonal antisera were raised (rabbit A, 50 kDa band; and rabbit B, 46 kDa band) at multiple sites. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the incorporation of 1000 cpm of radioactive sugar into product(s) under the conditions of the assay. Galactosyltransferase was determined as described (25). The activity of 5'-nucleotidase was assayed according to Aronson and Touster (26) measuring the inorganic phosphate formed from ATP. 80 μl of ATP (25 μCi/mmol) were added to the reaction mixture for 15 min. After gel electrophoresis, the gel was dried and subjected to immunoblotting conditions with 1:1000 diluted antiserum.
after clotting of the blood were used directly for immunoinhibition of GlcNAc-1-P-transferase activity. To obtain a better background on the Western blots, both antisera were passed over columns of CM-Affi-Gel (28) and further subjected to precipitation with ammonium sulfate (40% saturation). The precipitated globulins were dissolved in phosphate-buffered saline to a protein concentration of 20 mg/ml. Addition of 1% Nonidet P-40 yielded a titer which was at least 10 times higher than that of anti-A. Since anti-A and anti-B recognized similar epitopes in the two protein bands of the purified enzyme (see "Results"), anti-B antibodies were used for a number of experiments as stated.

**Immunoinhibition**—The antisera to protein band A and protein band B were serially diluted with phosphate-buffered saline containing bovine serum albumin (1 mg/ml) and incubated for 4 h at 4 °C with a constant amount of the solubilized enzyme preparation in buffer A in a 1.5-ml Eppendorf tube. At the end of incubation, protein A-Sepharose, washed and equilibrated with buffer A, was added (200 μl of wet gel) and mixed end-over-end on a rotator for 2 h at 4 °C. The mixture was centrifuged, and GlcNAc-1-P-transferase activity was assayed in the supernatant. Control experiments with preimmune serum were carried out under identical conditions.

**Immunoprecipitation**—The specificity of the partially purified antibody preparations was checked by Western blotting as described (28). Additionally, 5 μg of the crude solubilized enzyme was iodinated with IODO-Beads and Na2125I following the instructions of the manufacturer (Pierce). The iodinated proteins (2 × 106 cpm) were diluted to 600 μl with immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 0.5% sprotinin, 1.0% Nonidet P-40, 2 mM EDTA, and 0.15 M NaCl), and 10 μl of anti-B antibodies were added. The mixture was incubated at 4 °C for 2 h on a rotator. The residual ether was evaporated under a gentle stream of Nz; the mixture was centrifuged, and GlcNAc-1-P-transferase with enzyme preparation from different concentrations of Nonidet P-40, GlcNAc-1-P-transferase was optimally solubilized at a detergent concentration of 0.25%; higher concentrations of the detergent were inhibitory to the enzyme.

The solubilized enzyme was extremely unstable and lost most of its activity within 12 h at 4 °C. In attempting a purification of this enzyme, it was necessary to stabilize it for at least a week at 4 °C. Kaushal and Elbein (16) stabilized the enzyme solubilized from pig aorta microsomes with 20% glycerol and 20 μg of phosphatidylglycerol/ml. While the stability of the solubilized bovine mammary enzyme improved considerably with the addition of these compounds, it was still not enough to permit purification. After trying a variety of potential stabilizing agents, the best results were obtained by including 20% glycerol, 20 μg of phosphatidylglycerol/ml, 5 μM dolichol phosphate, and 2.5 μM UDP-GlcNAc in the solubilization buffer. The crude solubilized enzyme retained nearly 90% of its activity after 7 days at 4 °C under these conditions. A preliminary standardization of the purification protocol established two facts: 1) UDP-GlcNAc (2.5 μM) was essential for an extended stability of the enzyme; 2) the simultaneous presence of the detergent and the salt used for gradient chromatography was extremely detrimental to the enzyme. Efforts to therefore minimize the presence of salt and detergent together in the buffers during purification. On the other hand, if the detergent was not included in the buffer for equilibrating and eluting the hydroxylapatite column, active enzyme could not be eluted from this matrix. Further, it was necessary to collect the enzyme eluted from hydroxylapatite into polypropylene tubes containing glycerol, phosphatidylglycerol, and dolichol phosphate (as given under "Experimental Procedures"). The dilute concentration of protein at this stage could have caused a rapid denaturation of the enzyme. However, inclusion of bovine serum albumin as carrier did not improve the stability of the enzyme. The purification profile of GlcNAc-1-P-transferase is presented in Table 1, and elution of enzyme activity during different chromatographic steps is shown in Fig. 1, panels A-D. The pools of enzymatically active fractions obtained at different stages of purification were also monitored for the activity of two other glycosyltransferases of the dolichol cycle, viz. GDP-Man:Man9Glcα1-P:mannosyltransferase and UDP-Glc:Glcα1-P glucosyltransferase. Clearly, the hydroxylapatite chromatography step is critical to the separation of GlcNAc-1-P-transferase from the other two transferases of the dolichol cycle (Table II).

The purified enzyme eluting from the hydroxylapatite column was examined by SDS-PAGE under reducing conditions (Fig. 2). Both silver and Coomassie Blue staining revealed two bands of molecular mass 50 kDa (band A) and 46 kDa (band B). The staining intensity of bands A and B coincided

### Table I

| Purification steps | Volume | Total protein | Total activity | Specific activity | Purification Yield |
|-------------------|--------|---------------|----------------|------------------|---------------------|
|                   | ml     | mg            | mg/1000 units | μg/mg            | -fold %             |
| Solubilized enzyme| 250    | 620           | 195            | 544              | 6.6                 |
| (NH4)2SO4 precipitate | 35    | 335           | 181            | 544              | 4.5                 |
| Sepharose C-300   | 180    | 162           | 126            | 778              | 4.5                 |
| DEAE-TSK          | 90     | 53            | 44             | 830              | 6.9                 |
| Hydroxylapatite   | 10     | 0.35          | 2.9            | 8285             | 0.6                 |

***GlcNAc-1-P-transferase in the Mammary Gland***

We previously observed that glycosyltransferases for the biosynthesis of dolichol-linked intermediates up to Man9GlcNAc9 and Glc-P-Dol in the bovine mammary microsomes could be readily solubilized with the nonionic detergent, Nonidet P-40 (31). When these microsomes were treated with different concentrations of Nonidet P-40, GlcNAc-1-P-transferase was optimally solubilized at a detergent concentration of 0.25%; higher concentrations of the detergent were inhibitory to the enzyme.

**RESULTS**

**Purification of the Transferase**

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| Hydroxylapatite   | 10     | 0.35          | 2.9            | 8285             | 0.6                 |
GlcNAc-1-P-transferase in the Mammary Gland

FIG. 1. Chromatographic steps during the purification of GlcNAc-1-P-transferase. The 50% ammonium sulfate precipitate was dissolved in buffer A and applied to a column of Sephacryl S-300

TABLE II

| Purification steps | GlcNAc-1-P-transferase  | Mannosyl-transferase  | Glucosyl-transferase |
|--------------------|------------------------|-----------------------|----------------------|
| units/mg           |                        |                       |                      |
| Microsomes         | 120                    | 66                    | 8                    |
| Solubilized enzyme | 314                    | 97                    | 8                    |
| Sephacryl S-300    | 778                    | 25                    | 3                    |
| DEAE-TSK           | 830                    | 2                     | 2                    |
| Hydroxylapatite    | 8285                   | 0                     | 0                    |

with the peak of enzyme activity; further, band B stained more intensely than band A (Figs. 1D and 2).

The bands A and B were individually cut out of the slab gel with attention paid to avoid any cross-contamination. Polyclonal antibodies were raised against these bands in rabbits. Incubation of either of the antisera with the solubilized bovine mammary microsomes inhibits GlcNAc-1-P-transferase activity (Fig. 3, A and B). Control experiments with preimmune serum did not show significant inhibition of enzyme activity. To eliminate the possibility that inhibition of the enzyme activity in the previous experiment was caused by a nonspecific binding of the enzyme to immune complexes, the solubilized microsomes were iodinated with Na\(^{125}\)I and precipitated separately with anti-B antibodies. The precipitated proteins were analyzed by SDS-PAGE autoradiography. Protein bands corresponding to 70, 50, and 46 kDa were observed (Fig. 4). Controls involving immunoprecipitation with preimmune serum and purified GlcNAc-1-P-transferase added to inhibit competitively in the immunoprecipitation reaction did not show any of these bands. A few faster moving bands near the dye front were also seen in all the lanes. These bands could be attributed to background noise of the immunoprecipitation reaction.

On Western blot, partially purified anti-A as well as anti-B antibodies recognized both polypeptides A and B. The solubilized bovine mammary microsomal proteins were transblotted to nitrocellulose after electrophoresis at the concentration given in the legend to Fig. 5. The blots were probed (panel A). The column fractions were monitored for GlcNAc-1-P-transferase activity and protein concentration by the BCA dye binding method. Fractions corresponding to 320–410 ml of eluate were pooled and applied to a column of DEAE-TSK equilibrated with buffer B (panel B). The column was washed and eluted as described in the text. Individual fractions from the column were monitored for the transferase activity and dye protein concentration by the dye binding assay. Fractions comprising the 270–312 ml of eluate, containing most of the enzyme activity, were pooled, desalted on Bio-Gel P-2, and applied to a column of hydroxylapatite equilibrated with buffer A (panel C). The column was washed and eluted as described in the text. The column was monitored at A\(_{280}\) for protein elution, and individual fractions were assayed for the transferase activity. The high background at A\(_{280}\) is due to the presence of UV absorbents, e.g. o-phenanthroline, phenylmethylsulfonyl fluoride, and Nonidet P-40 in the elution buffer. Panel D shows SDS-PAGE of the polypeptides present in fractions under the peak of enzyme activity in panel C. The fractions marked 1, 2, ... , 9 were concentrated to 1.25 ml by ultrafiltration on PM-30; 70 \(\mu\)l of each fraction were mixed with 25 \(\mu\)l of 4 X Laemmli sample buffer and 5 \(\mu\)l of 8-mercaptoethanol. After boiling for 2 min, fractions were subjected to electrophoresis followed by staining with silver reagents.
**GlcNAc-1-P-transferase in the Mammary Gland**

**FIG. 2.** SDS-PAGE of purified GlcNAc-1-P-transferase. The purified enzyme after concentration of the pool of the peak fractions of enzymatic activity in Fig. 1C was electrophoresed in 7.5% acrylamide under reducing conditions and stained with silver reagents.

**Fig. 3.** Immunoinhibition of solubilized GlcNAc-1-P-transferase activity with antibodies. 150 μg of solubilized microsomal protein was incubated with different amounts of antiserum or preimmune serum as described under "Experimental Procedures." After precipitation with protein A-Sepharose, the activity of GlcNAc-1-P-transferase was assayed in the supernatant. Panel A, reaction with anti-B antiserum (●●●) and with preimmune serum (▲▲▲); panel B, reaction with anti-A antiserum (●●●) and with preimmune serum (▲▲▲).

**FIG. 4.** Immunoprecipitation of GlcNAc-1-P-transferase by partially purified anti-B antibodies. Solubilized microsomal proteins were iodinated with Na$I^{125}$I and immunoprecipitated with anti-B antibodies. The precipitated proteins were analyzed by SDS-PAGE followed by autoradiography. *Lane 1,* immunoprecipitation with anti-B antibodies; *lane 2,* 5 μg of purified GlcNAc-1-P-transferase was added to the $^{125}$I-labeled solubilized microsomal proteins before adding anti-B antibodies; *lane 3,* immunoprecipitation of the $^{125}$I-labeled solubilized microsomal proteins with preimmune serum.

**Fig. 5.** Western blot of GlcNAc-1-P-transferase. The numbers in parentheses correspond to the protein applied. *Lane 1,* Nonidet P-40-solubilized microsomes (300 μg) probed with anti-A antibodies; *lane 2,* purified GlcNAc-1-P-transferase (2 μg) probed with anti-A antibodies; *lane 3,* Nonidet P-40-solubilized microsomes (300 μg) probed with anti-B antibodies; *lane 4,* purified GlcNAc-1-P-transferase (2 μg) probed with anti-B antibodies.
with either anti-A or anti-B antibodies; polypeptides corresponding to bands A and B and the 70-kDa size were decorated (Fig. 5).

GlcNAc-1-P-transferase and other enzymes of the dolichol cycle have been shown to be localized in the rough endoplasmic reticulum compartment of the eukaryotic cells (1). To support further the identity of the purified GlcNAc-1-P-transferase, the lactating mammary gland was processed to obtain cell-free homogenate and fractions enriched in plasma membrane, Golgi, and rough endoplasmic reticulum. An analysis of these fractions for 5'-nucleotidase as a marker for the plasma membrane, UDP-Gal:GlcNAc-β1,4-galactosyltransferase as the marker for the Golgi and GlcNAc-1-P-transferase is shown in Fig. 6. The markers are enriched in different fractions as expected. A Western blot of the above fractions, solubilized microsomes, and the purified enzyme is shown in Fig. 7. The amount of protein used for each fraction is given in the figure legend. The results show that the anti-B antibodies specifically detect the 50- (band A) and 46-kDa (band B) polypeptides in the different fractions. Additionally, a faint band corresponding to 70-kDa size is also visible in the lane of solubilized microsomes. The staining intensity of these bands is consistent with the specific activity of GlcNAc-1-P-transferase in these fractions.

The data obtained thus far were consistent with either of the following interpretations: 1) polypeptides A and B represent closely related subunits of the enzyme with 70 kDa band as a precursor form of the enzyme; 2) bands A and B represent partially proteolyzed forms of the enzyme that has an apparent molecular mass of 70 kDa. The latter interpretation is consistent with the observation that band B stains more intensely than band A. To evaluate a relatedness of polypeptides A (50 kDa) and B (46 kDa), the purified enzyme was subjected to SDS-PAGE; the gel areas corresponding to the polypeptides A and B were cut out and treated for peptide mapping analysis with N-chlorosuccinimide (Fig. 8). It is clear from the pattern of peptide fragments obtained that both polypeptide A and polypeptide B are highly homologous.

**Product Analysis**

Since UDP-GlcNAc serves as the donor for the biosynthesis of both GlcNAc-P-P-Dol and GlcNAcβ1→4GlcNAc-P-P-Dol

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**Fig. 6.** The specific activities of GlcNAc-1-P-transferase (■), galactosyltransferase (□), and 5'-nucleotidase (□) in different subcellular fractions of the mammary tissue. Details of the experimental protocol are given in the text. Homo, homogenate; PM, plasma membrane; RER, rough endoplasmic reticulum.

**Fig. 7.** Western blot of proteins in different subcellular fractions probed with anti-β antibodies. Lane 1, homogenate (375 μg); lane 2, Golgi (60 μg); lane 3, plasma membrane (60 μg); lane 4, rough endoplasmic reticulum (60 μg); lane 5, pure GlcNAc-1-P-transferase (2 μg); lane 6, Nonidet P-40-solubilized microsomes (300 μg). Other details are in the text.

**Fig. 8.** Peptide mapping analysis of protein bands A (lane 1) and B (lane 2) of GlcNAc-1-P-transferase. Details of experimental protocol are given in the text.
by UDP-GlcNAc:Dol-P GlcNAc-1-P-transferase and UDP-GlcNAcGlcNAc-P-Dol β1,4GlcNAc-transferase (GlcNActransferase), respectively, it was important to know if the latter enzyme activity was removed during purification of GlcNAc-1-P-transferase. The labeled GlcNAc-lipid products obtained by the enzyme reaction after each step of purification were isolated and characterized. The labeled glycolipid(s) eluted from columns of DEAE-cellulose identically to the standards GlcNAc-P-P-Dol and GlcNAcβ1→4GlcNAc-P-P-Dol (not shown). Upon mild acid hydrolysis, the glycolipids released >90% of the radioactivity in water-soluble products. The radiolabeled products in the aqueous phase were analyzed by paper chromatography (22). As shown in Fig. 9, the microsomes, solubilized enzyme, and the fractions eluting from Sephacryl S-300 column catalyzed for formation of both GlcNAc- and GlcNAcβ1→4GlcNAc-P-P-Dol. The enzyme preparations after the DEAE-TSK and the hydroxylapatite chromatography steps gave GlcNAc-P-P-Dol as the sole product. Thus the purified enzyme was devoid of any detectable UDP-GlcNAcGlcNAc-P-Dol β1,4GlcNAc-transferase.

Characteristics of GlcNAc-1-P-transferase

Molecular Mass—Due to extreme instability, it was not possible to determine the molecular weight of the purified enzyme after hydroxylapatite chromatography. For this purpose, the more stable, partially purified enzyme from the DEAE-TSK column was applied to a calibrated column (2.5 × 90 cm) of Sephacryl S-300 packed in 0.1 M KCl-containing buffer B. The enzyme eluted from this column with an apparent mass of ≈330–360 kDa. The possibility that this large molecular mass of the enzyme could be due to aggregation of the hydrophobic, membrane-derived enzyme rather than resulting from a subunit interaction, cannot be ruled out.

Activation by Man-P-Dol—Man-P-Dol has been shown to stimulate the biosynthesis of GlcNAc-P-P-Dol and (GlcNAc)₆-P-P-Dol in microsomes from several different sources (17–19). Similarly, the partially purified GlcNAc-1-P-transferase from pig aorta was also shown to be stimulated by the addition of Man-P-Dol in the incubation mixture (16). Due to lack of availability, an activation of purified GlcNAc-1-P-transferase by Man-P-Dol had not been examined. We studied the effect of Man-P-Dol and Glc-P-Dol on the GlcNAc-1-P-transferase activity in bovine mammary microsomes as well as the purified enzyme (Fig. 10). A nearly 10-fold stimulation of enzyme activity was observed by preincu-

![Fig. 9](image-url)

**Fig. 9.** Analysis of products of GlcNAc-1-P-transferase obtained with enzymatically active fractions or pools at different stages of purification. Approximately 30,000–250,000 cpm of the water-soluble radioactive sugars released after mild acid hydrolysis of the product(s) obtained from enzyme reactions were subjected to paper chromatography in butanol/pyridine/water, 4:3:4, for 16 h. The paper chromatograms were cut into 1-cm segments and counted for radioactivity (22). Arrows indicate the migration positions of standards: 1, GlcNAc; 2, GlcNAcβ1→4GlcNAc. Panel A, microsomal fraction; panel B, solubilized enzyme; panel C, enzyme fractions from Sephacryl S-300 column; panel D, enzyme fractions from DEAE-TSK column; panel E, enzyme fractions from hydroxylapatite column.

![Fig. 10](image-url)

**Fig. 10.** Effect of Man-P-Dol and Glc-P-Dol on GlcNAc-1-P-transferase activity. Different amounts of Man-P-Dol and Glc-P-Dol dissolved in CHCl₃/CH₃OH, 2:1, were dried down under nitrogen and sonicated for 10 min after adding Dol-P (final concentration, 50 μM) and incubation mixture. Preincubation of these mixtures with 300 μg of microsomal protein or 4 μg of hydroxylapatite-purified enzyme was conducted for 10 min at room temperature. The reactions were initiated by the addition of 1 μCi of UDP-[³H]GlcNAc in a final volume of 0.1 ml and conducted for 10 min at 37 °C. After incubation for 10 min at 37 °C, the reactions were stopped with CHCl₃/CH₃OH, 2:1, and processed to obtain the chloroform-methanol-soluble products (22). Panel A, assay with microsomal preparation; panel B, assay with purified GlcNAc-1-P-transferase. The symbols represent activation with Man-P-Dol (●●●) and Glc-P-Dol (▲▲▲).
bating microsomes with Man-P-Dol (Fig. 10A); similar results were obtained with the purified enzyme preparation (Fig. 10B). However, Glc-P-Dol failed to stimulate the enzyme activity in either preparation. The only difference between Man-P-Dol and Glc-P-Dol is the configuration of the hydroxyl group on the second carbon of the sugar moiety within these glycolipids. These results show that the activation of the enzyme by Man-P-Dol is highly specific.

Other Properties—The pH optimum of the purified transference was found to be 7.4-7.6. Similar values have been reported for the microsomal enzyme in different tissues and the partially purified enzyme from pig aorta. The enzyme showed a requirement for divalent cations; maximal stimulation was observed with Mn$^{2+}$ and Mg$^{2+}$. Heavy metal ions, especially Hg$^{2+}$, were potent inhibitors of the enzyme activity. This inhibition could be partially reversed by diethiothreitol. Sulfhydryl reagents p-chloromercuribenzoate and iodoacetamide were strong inhibitors of the enzyme. Among various nucleotides and their derivatives, UDP, UTP, UDP-Glc, UDP-hexameramine, and UDP-xylose, were found to be inhibitory to the enzyme. Tunicamycin and dicyclohexylcarbodiimide were also potent inhibitors of the enzyme. Both bands A and B of the purified enzyme did not stain with periodic acid Schiff base. Neither the crude enzyme activity in the solubilized microsomes nor the purified enzyme could be bound to concanavalin A-Sepharose. Further, the purified enzyme was resistant to digestion with eitherendo-$eta$-N-acetylglucosaminidase H or F. These results indicate that GlcNAc-1-P-transerase is not a glycoprotein of the asparagine-linked type (data not shown).

The apparent $K_{m}$ values for UDP-GlcNAc and Dol-P were found to be 4.5 and 16 $\mu$M, respectively. At higher concentrations, Dol-P inhibited the enzyme activity; a similar observation has been reported for the partially purified glucosyltransferase preparation from murine plasmacytoma (32). The apparent $K_{m}$ values for UDP-GlcNAc and Dol-P were found to be 4.5 and 16 $\mu$M, respectively. At higher concentrations, Dol-P inhibited the enzyme activity; a similar observation has been reported for the partially purified glucosyltransferase preparation from murine plasmacytoma (32).

**DISCUSSION**

This study has focused on the purification and characterization of GlcNAc-1-P-transerase that catalyzes the following reaction:

$$\text{UDP-GlcNAc} + \text{Dol-P} \xrightarrow{\text{Mn}^{2+} \text{ or } \text{Mg}^{2+}} \text{GlcNAc-P-P-Dol} + \text{UMP}$$

As the first enzyme in the complex pathway of dolichol-linked assembly of the precursor oligosaccharide for the biosynthesis of asparagine-linked glycoproteins, this enzyme controls the flux of carbohydrate for the overall assembly of these conjugated proteins. Further, it could be the pacemaker enzyme for the entire sequence of reactions constituting the dolichol cycle. GlcNAc-1-P-transerase has been studied in microsomes and solubilized membrane preparations of a number of tissues (2-9, 15, 16). The extreme instability of the enzyme has hampered any extensive purification in different systems. Kaushal and Elbein (16) achieved a partial purification of the pig aorta enzyme after stabilization with glycerol and phosphatidylglycerol. The bovine mammary tissue was chosen for this study since this gland possesses a remarkable secretory capacity during lactation and is the target of a variety of hormones for its growth and differentiation. Among the proteins secreted into milk, $\alpha$-lactalbumin and transferrin are asparagine-linked glycoproteins (33). Several proteins of the milk fat globule membrane, believed to be derived from the apical plasma membrane of the mammary epithelial cells, also possess asparagine-linked oligosaccharides (34).

The work presented here provides a compelling evidence that the GlcNAc-1-P-transerase has been isolated to a high degree of purity. The final preparation contains two bands of 50 kDa (band A) and 46 kDa (band B). Several lines of evidence indicated that bands A and B were closely related polypeptides of the enzyme. First, different preparations of the enzyme gave the same two bands at the final step. The intensity of these bands on SDS-PAGE gels corresponded to the peak of enzyme activity eluting from the hydroxylapatite column. There appeared to be a gradual conversion of band A into band B if the purification was delayed, especially beyond chromatography on the DEAE-TSK column. Also, the band B stained more intensely than band A. Second, partially purified antibodies against either band A or band B recognized both polypeptides on the Western blots of purified enzyme as well as the solubilized microsomal proteins. This showed that bands A and B have common epitopes. Third, peptide mapping analysis by treatment with N-chlorosuccinimide, a reagent that leaves the tryptophanyl peptide linkages, gave very similar fragments for both bands A and B. Fourth, antisera against either of the bands A and B inhibited the enzyme activity in the immunoinhibition reaction in a dose-dependent manner.

Interestingly, in addition to recognizing the 50- and 46-kDa polypeptides in solubilized microsomal proteins, both partially purified anti-A and anti-B antibodies also gave immunological reaction with a 70-kDa polypeptide on the Western blots. It is reasonable to assume that the 70-kDa polypeptide is a precursor form of GlcNAc-1-P-transerase; alternatively, this polypeptide, representing either the native enzyme or its subunit, is rapidly proteolyzed to smaller peptides of 50 and 46 kDa during purification despite the presence of anti-proteases against serine type of proteolytic enzymes that were included in all buffers used in this study. The possibility that the 70-kDa polypeptide represents another protein (a glycosyltransferase) with epitopes similar to those of GlcNAc-1-P-transerase cannot be ruled out.

Several Golgi-localized glycosyltransferases that directly transfer the sugars from donor nucleotide-sugars to acceptor polypeptides have been purified to homogeneity. These enzymes required several thousand to 100,000 or higher-fold purification before yielding homogeneous preparations. It is possible that GlcNAc-1-P-transerase has been greatly inactivated during purification. The low factor to achieve the purification of the enzyme warrants some discussion, since so far there is no precedent for an extensive purification of any of the glycosyltransferases of the dolichol cycle.

As mentioned earlier, the coelution of only two protein bands of 50 and 46 kDa whose intensity parallels the peak of GlcNAc-1-P-transerase activity coupled with immunoinhibition of enzyme activity by antisera prepared against these bands is perhaps as strong an evidence as can be obtained to validate the purification and characterization data. It is difficult to conclude that both 50 and the 46 kDa bands represent enzymatically active polypeptides. The extreme instability of the enzyme and its likely gradual proteolysis during purification make it virtually impossible to state unequivocally the exact size of its native polypeptide(s). The data provided in Figs. 6 and 7 provide additional support that the protein purified in this study is GlcNAc-1-P-transerase.

It must be emphasized that the quantitation of GlcNAc-1-P-transerase in this study at different stages of purification is most likely a gross underestimate of the actual -fold purification and has little significance in light of the experimental protocol employed. The specific activity of the enzyme in the crude microsomes is an overestimate due to the presence of the second enzyme of the dolichol cycle, i.e. $\beta$4GlcNAc-transferase. The inclusion of 25 $\mu$M unlabeled UDP-GlcNAc was absolutely necessary to stabilize the enzyme activity.
during purification. During chromatography on DEAE-TSK, there was a significant separation of other proteins, without much increase in the specific activity of the enzyme. If there was a selective binding and elution of UDP-GlcNAc along with the enzyme, then it would raise the concentration of UDP-GlcNAc in the eluted enzyme fractions. To test this possibility, a portion of the peak fractions of enzyme activity eluted from the DEAE-TSK column was boiled, and added to the solubilized enzyme. A strong inhibition of activity of the solubilized enzyme was observed (data not shown). A progressive enrichment of enzyme fractions with "tightly bound" unlabeled UDP-GlcNAc would lower the radiospecific activity of the substrate in assays for activity determination.

Despite the initial stabilization at the solubilization stage, the enzyme cannot withstand the simultaneous presence of both detergent and the salt used for gradient elution. Any denaturation of the enzyme during purification would seriously interfere in calculations of fold purification of the enzyme. A proteolytic degradation of the enzyme during purification could also significantly affect the activity of the enzyme.

The partially purified enzyme from pig aorta was stimulated by a heat-stable factor that separated from the enzymatically active fractions during chromatography on DEAE-cellulose (16). We have been unable to find such an activator in the bovine mammary microsomal preparation. We added boiled solubilized microsomal proteins as well as different fractions (supernatant from (NH₄)₂SO₄ precipitation, different fractions eluted from Sephacryl S-300, DEAE-TSK, and hydroxyapatite columns) to the enzyme at the successive stages of purification; however, no activation in enzyme activity was obtained. A lack of activator in this study may relate to an inherent difference in the aorta and the mammary enzymes.

The results of a previous study (22) from this laboratory and the data on product analysis show that the transferase purified here catalyzes the synthesis of GlcNAc-P-P-dolichol. A number of properties of the enzyme were characterized. These are very similar to the characteristics of microsomal GlcNAc-1-P-transferase studied in other systems (2-9, 15, 16). The enhanced activity of the purified enzyme by Man-P-Dol is also consistent with the results of other studies conducted with the microsomal or partially purified enzyme. This activation is rather remarkable and does not seem to be saturated at the concentrations of Man-P-Dol used. We earlier reported the lack of any measurable activity of a phosphodiesterase or a pyrophosphatase in bovine mammary microsomes that could degrade dolichol- or nucleotide-linked sugars (22). Further, we routinely included AMP, adenosine 5'-[(α,β-methylene)triphosphate and 2,3-dimercapto-1-propanol, potent inhibitors of a sugar-nucleotide pyrophosphatase (35, 36), in all assays. Thus the specific activation by Man-P-Dol may not be attributed to a competitive inhibition of hydrolysis that would degrade the product or the substrate of the enzyme. The interesting feature reported here is the total lack of any stimulation of the purified enzyme by Glc-P-Dol and how it might relate to the overall structure of the native enzyme. Assuming an average chain length of C₁₀₀ for the dolichol moiety, the molecular weight of these glycolipids would be 1549. The only difference between Man-P-Dol and Glc-P-Dol is the disposition of the hydroxyl group on the second carbon of the sugar residue. Thus, the binding site for Man-P-Dol on the enzyme is extremely specific for the configuration at just this carbon atom in such an effector.

With the availability of highly specific polyclonal antibodies that recognize both the native as well as the denatured forms of the enzyme, it should be possible to study the biosynthesis of this enzyme in a cell culture system and establish the nature of the 70-kDa polypeptide as it relates to this enzyme. These antibodies may also serve as potential immunological probes for the cloning of this enzyme and studying its regulation in the mammary gland. Our present experiments are designed to investigate these avenues.

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