Regulation of the Biosynthesis of N-Acetylgalactosaminylpyrophosphoryldolichol, Feedback and Product Inhibition*

(Received for publication, August 10, 1999, and in revised form, September 16, 1999)

Edward L. Kean§§, Zenglu Wei†, Vernon E. Anderson§§, Nanjing Zhang, and Lawrence M. Sayre¶
From the Departments of  §Ophthalmology,  ¶Biochemistry, and  §§Chemistry, Case Western Reserve University, Cleveland, Ohio 44106

The assembly of the core oligosaccharide region of asparagine-linked glycoproteins proceeds by means of the dolichol pathway. The first step of this pathway, the reaction of dolichol phosphate with UDP-GlcNAc to form N-acetylgalactosaminylpyrophosphoryldolichol (GlcNAc-P-P-dolichol), is under investigation as a possible site of metabolic regulation. This report describes feedback inhibition of this reaction by the second intermediate of the pathway, N-acetylgalactosaminyl-N-acetylgalactosaminylpyrophosphoryldolichol (GlcNAc-GlcNAc-P-P-dolichol), and product inhibition by GlcNAc-P-P-dolichol itself. These influences were revealed when the reactions were carried out in the presence of showdomycin, a nucleoside antibiotic, present at concentrations that block the de novo formation of GlcNAc-GlcNAc-P-P-dolichol but not that of GlcNAc-P-P-dolichol. The apparent $K_i$ values for GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol under basal conditions were 4.4 and 2.8 M, respectively. Inhibition was also observed under conditions where mannosyl-P-dolichol (Man-P-dol) stimulated the biosynthesis of GlcNAc-P-P-dolichol; the apparent $K_i$ values for GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol were 2.2 and 11 M, respectively. Kinetic analysis of the types of inhibition indicated competitive inhibition by GlcNAc-P-P-dolichol toward the substrate UDP-GlcNAc and non-competitive inhibition toward dolichol phosphate. Inhibition by GlcNAc-GlcNAc-P-P-dolichol was uncompetitive toward UDP-GlcNAc and competitive toward dolichol phosphate. A model is presented for the kinetic mechanism of the synthesis of GlcNAc-P-P-dolichol. GlcNAc-P-P-dolichol also exerts a stimulatory effect on the biosynthesis of Man-P-dol, i.e. a reciprocal relationship to that previously observed between these two intermediates of the dolichol pathway. This network of inhibitory and stimulatory influences may be aspects of metabolic control of the pathway and thus of glycoprotein biosynthesis in general.

It has been well established that the dolichol pathway is the means whereby the core region of asparagine-linked glycoproteins is assembled (see review, Ref. 1). Our understanding of the mechanisms that regulate this complex series of reactions, however, is still limited. To this end we have directed our attention to the initial reaction of the pathway, the reaction between dolichol phosphate and UDP-GlcNAc producing GlcNAc-P-P-dolichol, catalyzed by the enzyme, UDP-GlcNAc-dolichyl-phosphate N-acetylgalactosamine 1-phosphate transferase (GPT-1). Factors that modulate the formation of GlcNAc-P-P-dolichol could have an effect on the rate of synthesis of the other intermediates of the dolichol pathway and thus influence nascent glycoprotein biosynthesis in general. Several factors have previously been described that could have a regulatory influence on this reaction as follows: hormonal effects (2), genetic factors (3), and topography of enzyme and substrates (4–6). Previous studies from this laboratory and others (7–15) have also revealed that another intermediate of the pathway, mannosyl-P-dolichol (Man-P-dol), acts as an allosteric activator of GPT-1, resulting in the stimulation of GlcNAc-P-P-Dol synthesis. A reciprocal relationship has now been revealed whereby Man-P-Dol formation is stimulated by GlcNAc-P-P-dolichol. The present study has revealed other potential aspects of regulation of the initial reaction of the dolichol pathway. Feedback inhibition of the biosynthesis of GlcNAc-P-P-dolichol was demonstrated by the second intermediate of the pathway, GlcNAc-GlcNAc-P-P-dolichol. The formation of the latter compound is catalyzed by a separate GlcNAc-transferase, UDP-GlcNAc:GlcNAc-P-P-dolichol, N-acetylgalactosamine transferase (GT-2), the kinetics of which have recently been described (16). Although the reversibility of GPT-1 has previously been demonstrated (17, 18), the present report also described the kinetics of inhibition by GlcNAc-P-P-dolichol of its own synthesis. We have examined the effect of these inhibitory influences on the biosynthesis of GlcNAc-P-P-dolichol at the basal level and under stimulatory conditions in the presence of Man-P-dol. On the basis of these relationships a model is suggested as a mechanism of action of UDP-GlcNAc:dolichol phosphate, GlcNAc-1-phosphate transferase.

EXPERIMENTAL PROCEDURES

Enzyme Preparation, Chemicals

Microsomes were prepared from the retinas of 15–16-day-old embryonic chicks as described previously (10). Purified, recombinant yeast mannosyl-P-dolichol synthase was obtained from Dr. John Schutzbach.

1 The abbreviations used are: GlcNAc-P-P-Dol, N-acetylgalactosaminylpyrophosphoryldolichol; GlcNAc-GlcNAc-P-P-dolichol, N-acetylgalactosaminyl-N-acetylgalactosaminylpyrophosphoryldolichol; Man-P-dol, mannosylphosphoryldolichol; Tes, 2-[(tris(hydroxymethyl)methyl)amino]ethanesulfonic acid; GPT-1, UDP-GlcNAc:dolichyl-phosphate N-acetylgalactosamine 1-phosphate transferase; GT-2, UDP-GlcNAc:GlcNAc-P-P-dolichol, N-acetylgalactosamine transferase; C/M, chloroform/methanol.

2 Preliminary reports of some of these studies have been made (Kean, E. L., Niu, N., and Imperiali, B. (1996) Glycobiol. 6, 740; Kean, E. L. (1997) Glycoconjug. J. 14, (suppl) S35).
Dolichol phosphate was purchased from Sigma. UDP-[14C]GlcNAc and GDP-[14C]mannose were purchased from NEI Life Science Products. UDP-[14C]GlcNAc, UDP-[14C]GlcNAc, and GDP-[3H]mannose were purchased from American Radiochemicals, Inc. d(-)-Showdomyycin was obtained from Dr. Sung Ho Kang, Department of Chemistry, Korea Advanced Institute of Science and Technology, Taejon, Korea. N-Benzyl-2-deoxyshowdomyycin was obtained from Dr. R. S. Hossman, Department of Chemistry and Biochemistry, University of Maryland Baltimore County, Baltimore.

Preparation of GlcNAc-P-P-dolichol

Enzymatic Synthesis—Large scale preparations were performed by incubating microsomes from the retina of the embryonic chick with UDP-[14C]GlcNAc and dolichol phosphate, as described previously (10). The incubations were performed in the presence also of Man-P-Dol and the antibiotic, showdomyycin. As described previously, the former stimulates the production of GlcNAc-P-GlcNAc-P-P-dolichol and also brings about an increase in the production of the mono-GlcNAc derivative (19). The product was isolated by solvent partitioning according to the procedure of Folch et al. (20) and purified by chromatography on DEAE-cellulose, as described previously (10). Its concentration was determined by Dionex chromatography, as described below.

Chemical Synthesis—By using dolichol kindly provided by Dr. Tadeusz Cichoñacki of the Institute of Biochemistry and Biophysics, Warsaw, Poland, GlcNAc-P-P-dolichol was initially synthesized by the method of Imperiali and Zimmerman (21), which requires azeotropic drying (toluene or pyridine) at the stage of the oxalyl chloride-mediated activation of dolichol phosphate but which could not successfully be coupled directly to the protected GlcNAc-P. The latter intermediate could be obtained only by careful azeotropic drying of all commercial ingredients (including tetraethylammonium chloride and dibenzylphosphate) and use of freshly distilled solvents. In the published method (21), oxalyl chloride activation of dolichol phosphate generates the highly activated doli-chylphosphoryl dichloride, which actually is an intermediate in the POCl3-mediated preparation of dolichol phosphate but which could not successfully be coupled directly to the protected GlcNAc-P, and was instead hydrolyzed to dolichol phosphate, which was then purified and reactivated with oxalyl chloride. During this study, it was found that the requisite dolichylphosphoryl dichloride could in fact be generated from dolichol in a directly usable form using excess POCl3 in hexane, followed by a brief extraction with water to remove HOPOCl2, and then evaporation of hexane and remaining POCl3, under high vacuum. Following coupling, purification of the protected pyrophosphate prior to NaOMe-mediated deacetylation was achieved by silica gel 60 column chromatography using CHCl3/MeOH/H2O (65:25:4, by volume) as the eluant. These modifications, which avoid the need to isolate and purify dolichol phosphate, resulted in the best overall yield of GlcNAc-P-P-dolichol from dolichol.

The enzymatically and chemically synthesized GlcNAc-P-P-dolichol functioned in a similar manner in these studies.

Large Scale Preparation of Mannosyl-P-dolichol—Large scale preparations of [14C]Man-P-Dol were made by incubating dolichol phosphate, GDP-[14C]mannose (1.7 dpm/pmol), buffer, and metal ions as described previously (12) with extracts from cells, as described previously (10). As indicated by guide strips, the Man-P-dolichol region was scraped from the plates and recovered by elution of the gel in the cold with C/M (chloroform/methanol)/water (10:10:3) followed by GDP-[14C]mannose. After solvent partitioning by the procedure of Folch et al. (20), the concentration of [14C]Man-P-Dol was calculated from the specific activity of GDP-[14C]mannose used in its preparation.

Incubation Conditions and Assay, Kinetic Studies

GlcNAc Lipid Biosynthesis—Incubations were carried out for 10–20 min at 37 °C in the presence of dolichol phosphate (16–20 μmol), Triton X-100 (0.15%), UDP-[14C]GlcNAc (52 μCi; 169 dpm/pmol), MgCl2 (27 mM), showdomycin, or N-benzyl-2-deoxyshowdomycin, as indicated, and enzyme (0.2–0.25 mg of protein) in a total volume of 0.15 ml (basal conditions). Reactions were also carried out in the presence of Man-P-Dol (2.1–6.4 μmol) (stimulatory conditions). The incubations were performed in the absence or presence of exogenously added GlcNAc-P-P-dolichol or GlcNAc-GlcNAc-P-P-dolichol (as indicated in the tables and figures). The dolichol derivatives were evaporated to dryness with nitrogen, vortexed vigorously with 0.015 ml of 1.5% Triton X-100, after which the other components of the reaction mixture were added. The incubations, carried out at 37 °C, were started by the addition of the enzyme preparation and stopped by the addition of C/M (2.1). After solvent partitioning by the Folch procedure (20) the radioactivity in the washed lower phase was determined by scintillation spectrometry, as described previously (7, 10).

Feedback and Product Inhibition of GlcNAc-P-P-Dol Biosynthesis

The product was analyzed by scintillation spectrometry after Folch washing, as described above.

Dilutions of purified, full-length, recombinant yeast Man-P-Dol synthase (25) were made in a buffer containing 10% glycerol, 0.015 M Tris-HCl, pH 7.5, 1% Nonidet P-40, 2 mM dithiothreitol, and 2 mM bovine serum albumin (the latter acted to stabilize the enzyme upon dilution). Incubations were carried out at 37 °C for 10 min essentially as in the method of Schutzbach et al. (25) (in a medium containing Noni- det P-40 (0.5%), 5 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2.5 mM MgCl2, 0.25 mM EDTA, 5 mM dithiothreitol, 32 μM dolichol phosphate, GDP-[3H]Man (18 μM, 12–22 dpm/pmol) in the presence or absence of GlcNAc-P-P-dolichol (as indicated) and enzyme in a total volume of 0.15 ml. The products were analyzed by scintillation spectrometry after Folch washing, as above.

Dolichol Phosphorylation—After incubation followed by solvent partitioning as above, the material in the washed organic phase was evaporated to dryness and subjected to mild acid hydrolysis in 1 ml of 0.1 N HCl in 80% tetrahydrofuran for 10 min at 50 °C as previously described (28). After evaporation to dryness and redissolving in water, the material was applied to a column containing 0.5 ml each of AG-2-X8 (20–40 mesh) acetate and AG-50-X8 H+ (200–400 mesh), the column eluted with 20 ml of water, the eluate evaporated to dryness, and the residual material redissolved in water as described previously (16). To an aliquot was added 5 mM of fucose to serve as an early eluting reference marker and 10 nmol each of GlcNAc and GlcNAc-GlcNAc to serve as internal standards for analysis by high pH anion exchange chromatography (Dionex Corp., Sunnyvale, CA). The mixture was injected onto a CarboPac PA1 column (250 × 4 mm) with a CarboPac guard column (3 × 25 mm) and eluted isocratically with a mixture of 25% of 100 mM NaOH plus 75% 1 mM NaOH at a flow rate of 1 ml/min. The elution of the standards was followed by pulsed amperometric detection (high pH anion exchange chromatography-pulsed amperometric detection), and the products of the reactions by measuring their radioactivity by scintillation spectrometry of 0.5 ml fractions collected from the pulsed amperometric detection cell, as described previously (27).

Distribution of Radioactivity in the Glucosaminyi Residues of the Biosynthesized GlcNAc Lipids

The relative contributions of GPT-1 and G2-2 was investigated by analyzing the distribution of the tritium label in the GlcNAc residues of their respective products, GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol, as described previously (28, 29). In short, this involves the following procedures carried out sequentially. After incubation, the GlcNAc lipids extracted into the chloroform-rich layer after solvent partitioning are subjected to mild acid hydrolysis. GlcNAc and GlcNAc-GlcNAc thus formed are separated by paper chromatography, recovered from the chromatogram, and reduced with NaBH4. After mixed bed ion exchange chromatography, strong acid hydrolysis N-deacylates the products and cleaves chitobiose. The products are then subjected to high voltage paper electrophoresis in 1% sodium borate buffer, the electrophoretogram cut into 1-cm zones, and the radioactivity determined by scintillation spectrometry. The mobilities of standard [14C]GlcNH2OH, and [3H]GlcNH2OH were determined in the same manner. By these procedures glucosaminol was found to be present only in GlcNAc-P-P-dolichol after hydrolysis and glucosamine from the non-reducing end of GlcNAc-GlcNAc-P-P-dolichol after hydrolysis and glucosaminol from the reducing end.

3 J. S. Schutzbach, personal communication.
Other Analytic Procedures

The concentration of GlcNAc-P-P-dolichol was determined after mild acid hydrolysis, as above, by quantitative Dionex chromatography of the liberated GlcNAc and by the Morgan-Elson reaction as described by Reissig et al. (30). The concentration of GlcNAc-GlcNAc-P-P-dolichol, provided by Dr. B. Imperiali, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA, was determined by Dionex chromatography of N,N'-diacetylchitobiose liberated by mild acid hydrolysis. The concentration of dolichol phosphate and the GlcNAc-P-P-dolichol used in the early phases of this work (provided by Dr. Imperiali) was determined by analysis for total phosphate as described previously (10). Total microsomal phospholipids were determined in a similar manner after solvent partitioning (20) of retina microsomes. Thin layer chromatography was performed using 20 x 20-cm glass plates precoated with a 0.25- or 0.50-mm layer of Silica Gel 60 without fluorescent indicator. The following solvent systems were used: 1) chloroform/methanol/acetic acid/water (25:15:4:2, by volume); 2) chloroform/methanol/water (65:25:4, by volume). The location of radioactive material was accomplished by measuring the radioactivity by scintillation spectrometry of 1 x 3-cm zones scraped from the chromatogram. The migration of non-radioactive material was detected by the anisaldehyde spray reagent or by exposure to iodine vapor, as described previously (24).

Rationale for Using Showdomycin

Incubations carried out using dolichol phosphate and UDP-[3H]GlcNAc as substrates would result in the formation of [3H]GlcNAc-P-P-dolichol and [3H]GlcNAc-GlcNAc-P-P-dolichol. Thus, in assays carried out by solvent partitioning, any effect added compounds might have specifically on the new synthesis of [3H]GlcNAc-P-P-dolichol would be masked by the accompanying formation of the labeled chitobiosyl product. This difficulty was resolved by carrying out the incubations in the presence of the nucleoside antibiotic, showdomycin, which inhibits the formation of the chitobiosyl compound, enhancing the formation of GlcNAc-P-P-dolichol (19). The N'-benzyl-2-deoxy derivative of showdomycin was shown to have the same effect (19). Thus, using concentrations of the showdomycins that extensively inhibited the formation of GlcNAc-GlcNAc-P-P-dolichol, the effect of exogenously added non-radioactive GlcNAc-P-P-Dol on the de novo synthesis of the labeled compound could be readily determined.

Kinetics of Inhibition

Apparent \( K_i \) and \( V_{\text{max}} \) values were calculated from a non-linear least squares analysis of the data using GraFit (31) fit to an expression for general inhibition derived from Equation 1 for mixed inhibition with a constant substrate concentration.

\[
\frac{v (\text{inhibited})}{v (\text{uninhibited})} = \frac{V}{(1 + [I/K_i \text{ apparent}])}
\]

(Eq. 1)

Analyses of the types of inhibition were carried out in the following manner. The steady state kinetic data were fitted using the unweighted non-linear least squares method implemented in the computer program GraFit (31). Inhibition constants were determined by a global fit of the data from an entire experiment. The best fit of the data is one that minimizes the sum of the square of the observed and calculated velocities for an entire data set (32). The standard deviations of the data points were within 3% of the highest velocity. The data were initially fitted to Equation 2 for non-competitive or mixed inhibition. By using the criterion that if the standard error of the slope or intercept inhibition constants were over 50% of the fitted value, that parameter was excluded from the final fit. If so, the data were then fit to Equation 3 or Equation 4 for competitive and uncompetitive inhibition, respectively. The more restrictive mechanism was accepted if the \( \chi^2 \) squared value was not increased by omitting the poorly defined inhibition constant.

\[
v = \frac{V_{\text{max}} \cdot S}{K_n + S} + \frac{S}{1 + K_n / K_a}
\]

(Eq. 2)

\[
v = \frac{V_{\text{max}} \cdot S}{K_n + S} + \frac{S}{1 + K_n / K_a}
\]

(Eq. 3)

\[
v = \frac{V_{\text{max}} \cdot S}{K_n + S} + \frac{S}{1 + K_n / K_a}
\]

(Eq. 4)

RESULTS

Inhibition by GlcNAc-P-P-dolichol of GlcNAc Lipid Synthesis, Product Identification

Incubations were carried out under initial rate conditions for the yeast and retina microsomal enzymes. Apparent \( K_i \) and \( V_{\text{max}} \) values were calculated from Lineweaver-Burk double-reciprocal plots of the data after analysis by computer using the Kent program (BioMetals, Princeton, NJ).

Incubations were carried out under optimal conditions of GlcNAc lipid synthesis as described under “Experimental Procedures.” The presence or absence of exogenously added GlcNAc-P-P-dolichol. The incubation mixtures additionally contained showdomycin that inhibited 93--99% of the formation of GlcNAc-GlcNAc-P-P-dolichol. As shown in Fig. 1A, with increasing concentrations of GlcNAc-P-P-dolichol there was increasing inhibition (over 70%) of GlcNAc lipid synthesis. This effect on GlcNAc-P-P-dolichol biosynthesis occurred at the basal level and when the reaction was performed in the presence of Man-P-Dol shown previously to greatly stim-

![Image](329x430 to 533x729)

In Fig. 1A, inhibition of GlcNAc lipid synthesis by GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol under basal and stimulatory conditions. Incubations were carried out for 10--20 min at 37 °C in the presence of dolichol phosphate (18 μM), UDP-[3H]GlcNAc (51 μM, 165 dpm/pmol), showdomycin (0.13--0.7 mg/ml in different experiments), retina microsomes (0.23--0.27 mg of protein), Mg2+ (27 mM), Tes buffer (0.2 M, pH 7.5), Triton X-100 (0.15%) (basal conditions) or also in the presence of [14C]Man-P-Dol (3.7--4.8 μM, 1.7 dpm/pmol) (stimulatory conditions), and the concentrations of GlcNAc-GlcNAc-P-P-dolichol (A) or GlcNAc-GlcNAc-P-P-dolichol (B) indicated on the abscissa. The incubations and assays were carried out, and the incorporation of tritium into the products was determined by scintillation spectrometry as indicated under “Experimental Procedures,” using appropriate single or double labeling techniques. The lines were drawn after a non-linear least squares analysis of the data according to Equation 1 as described under “Experimental Procedures.” The data on the left ordinate refers to results obtained under basal conditions and on the right ordinate under stimulatory conditions in the presence of Man-P-Dol.
ulate its formation (10). In these studies, either under basal or stimulatory conditions, exogenously added GlcNAc-P-P-dolichol was present over a range from 2- to 86-fold over the \[^3H\]GlcNAc-P-P-dolichol formed in the absence of the inhibitor. (The curves in Fig. 1 were drawn in accord with an analysis of the data by Equation 1 as described under “Experimental Procedures.”)

The nature of the products formed under these conditions after analysis by Dionex chromatography is seen in Fig. 2. Fig. 2A shows a typical example of the distribution of the mono-GlcNAc and chitobiosyl products formed under basal conditions in the absence of showdomycin. As seen in Fig. 2B, under basal conditions in the presence of showdomycin, there was now extensive inhibition of the formation of the chitobiosyl product (solid line) and an increase in the formation of GlcNAc-P-P-dolichol (solid line) as described previously (19). When the reaction was carried out in the presence also of exogenously added GlcNAc-P-P-dolichol (18 \(\mu\)M), there was now an 86% decrease (diamond symbols) in the de novo formation of \[^3H\]GlcNAc-P-P-dolichol. Exogenously added GlcNAc-P-P-dolichol was present in 360-fold molar excess over the \[^3H\]GlcNAc-P-P-dolichol formed in its absence.

Similar results were obtained when the reactions were performed under stimulatory conditions, i.e. in the presence of Man-P-dolichol (Fig. 2C). The formation of GlcNAc-GlcNAc-P-P-dolichol under these conditions was reduced 97% in the presence of showdomycin (filled circles). When the reaction was carried out in the presence also of exogenously added GlcNAc-P-P-dolichol (18 \(\mu\)M), the formation of \[^3H\]GlcNAc-P-P-dolichol was decreased 88% (diamonds). The added GlcNAc-P-P-dolichol was present in 56-fold molar excess over that formed in its absence.

**Inhibition by GlcNAc-GlcNAc-P-P-dolichol, Product Identification**

The reaction between GlcNAc-P-P-dolichol and UDP-GlcNAc catalyzed by GT-2 results in the formation of the chitobiosyl derivative. As seen in Fig. 1B, when GlcNAc lipid synthesis was examined in the presence of exogenously added GlcNAc-GlcNAc-P-P-dolichol, inhibition of about 90% of that formed in the absence of the inhibitor was attained. As with the mono-GlcNAc derivative (Fig. 1A), inhibition was also induced and to a similar extent when the reaction was stimulated by the addition of Man-P-Dol as seen also in Fig. 1B. Shown in Fig. 2D is an identification of the products from experiments of this type using Dionex chromatography. The presence of showdomycin resulted in the essentially complete (96%) inhibition of the formation of the chitobiosyl derivative. When the incubation was performed in the presence also of exogenously added GlcNAc-GlcNAc-P-P-dolichol (diamond symbols), the formation of GlcNAc-P-P-dolichol was inhibited 94% of that formed in its absence. In this experiment, a 230-fold molar excess of GlcNAc-GlcNAc-P-P-Dol was added over the GlcNAc-P-P-dolichol formed in its absence.

The identification by Dionex chromatography of the products of the reactions under stimulatory conditions (+Man-P-dol) is seen in Fig. 2E. As under basal conditions the presence of showdomycin inhibited the formation of the chitobiosyl derivative 99% of that formed in its absence. In the presence also of exogenously added GlcNAc-GlcNAc-P-P-dolichol, the synthesis of GlcNAc-P-P-dolichol was inhibited 67% (diamonds). In this experiment a 42-fold molar excess of GlcNAc-GlcNAc-P-P-dolichol was added over the GlcNAc-P-P-dolichol formed in its absence.

A summary of the apparent \(K_i\) and \(V_{max}\) values generated by these inhibition studies is shown in Table I.

**Other Characteristics of the System, Inhibition Studies in the Absence of Showdomycin**

A series of experiments was performed to see if inhibition would also occur in the absence of showdomycin. As described previously, the simultaneous production of radioactive GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol would prevent merely looking for a loss of dpm in the organic phase after solvent partitioning as an assay to reveal the influence of added GlcNAc lipids on the formation of the mono-GlcNAc derivative. In order to investigate this without blocking the formation of GlcNAc-GlcNAc-P-P-dolichol (as affected by showdomycin in these studies), the isolation and analysis of the individual components of the reactions was performed as described under “Experimental Procedures.” The net accumulation of \[^3H\]GlcNAc-P-P-dolichol reflects the difference between its rate of formation and the rate of conversion to the chitobiosyl derivative. As seen in Table II, part A, at the basal level, in the absence of showdomycin, the addition of GlcNAc-GlcNAc-P-P-dolichol (19 \(\mu\)M) resulted in extensive net inhibition (74%) of GlcNAc-P-P-dolichol formation. Inhibition was also brought about by the chitobiosyl derivative under stimulatory conditions (+Man-P-dol) in which similar and extensive inhibition occurred in the presence or absence of the antibiotic as seen in Table II (part B, lines 1 and 2). Likewise, under stimulatory conditions in the absence of showdomycin where the presence of Man-P-Dol enhanced the uninhibited rate of \[^3H\]GlcNAc-P-P-dolichol formation about 7-fold (Table II, part D, lines a and b), exogenously added GlcNAc-P-P-dolichol inhibited the stimulatory response 62% (Table II, part D, lines b and c). The latter response, however, may not indicate a direct effect on GPT-1 at the basal level. Indeed, at the basal level using exogenously added GlcNAc-P-P-dolichol (18 \(\mu\)M) as the test substance, apparent stimulation rather than inhibition was detected in the absence of showdomycin, as seen in Table II (part C, line 1, lines a and b). Similar results were encountered using 2.9 and 9 \(\mu\)M GlcNAc-P-P-dolichol (data not shown). The reason for this apparent stimulation is, at least in part, accounted for by the isotope dilution effect of exogenously added unlabeled GlcNAc-P-P-dolichol (300–700-fold) preventing the conversion of \[^3H\]GlcNAc-P-P-dolichol to \[^3H\]GlcNAc-GlcNAc-P-P-dolichol. The chitobiosyl derivative formed under these conditions is predicted to be largely \[^3H\]GlcNAc-GlcNAc-P-P-dolichol. Because only a small fraction of the total GlcNAc-P-P-dolichol is converted to the chitobiosyl derivative, the isotope dilution results in almost all of the enzymatically synthesized \[^3H\]GlcNAc-P-P-dolichol being trapped. These effects can be confirmed by kinetic modeling of the reactions and numerical integration (33). We have modeled the GPT-1 and GT-2 system using the kinetic constants determined in this study to show that the increase in radioactivity observed in Table II, part C, line 1, lines a and b, is predicted by isotope dilution and fully consistent with inhibition of GPT-1 by GlcNAc-P-P-dolichol (data not shown).\(^4\) In addition to this theo-

---

\(^4\) Among the problems associated with studying enzyme kinetics in mixed micelle systems is whether the kinetic constants should be reported by concentration or as mole fractions. In these experiments, the large excess of Triton X-100 (about 2.5 mM) relative to the dolichol substrates/inhibitors or phospholipid added with the microsomes (0.24 \(\mu\)mol of total lipid P/mg protein) ensures that their mole fractions are proportional to their concentration, with a 1 \(\mu\)M concentration corresponding to a mole fraction of \(4 \times 10^{-4}\).

\(^5\) A copy of this material will be made available upon request to Dr. E. Kean.
Feedback and Product Inhibition of GlcNAC-P-P-Dol Biosynthesis

The kinetic values were calculated by Eq. 1 from a non-linear least squares analysis of the data plotted in Fig. 1, as described under “Experimental Procedures,” and are presented as the mean ± range (number of experiments), where appropriate.

| Inhibitor, kinetic property | Basal activity | Stimulated (+ Man-P-dol) |
|-----------------------------|----------------|------------------------|
| GlcNAC-P-P-dolichol         |                |                        |
| Apparent $K_i$ ($\mu$m)     |                |                        |
| $V_{\text{max}}$ (pmol/min/mg protein) |                |                        |
| GlcNAC-GlcNAC-P-P-dolichol  |                |                        |
| Apparent $K_i$ ($\mu$m)     |                |                        |
| $V_{\text{max}}$ (pmol/min/mg protein) |                |                        |

**Distribution of Label**

The trapping of $[^3\text{H}]$GlcNAC-P-P-dolichol by isotope dilution was examined by determining the distribution of radioactivity in the glucosaminyi residues of the GlcNAC lipids formed in the absence of showdomycin. The results from these experiments are presented in Fig. 3 showing the patterns obtained by high voltage paper electrophoresis after processing the products as described under “Experimental Procedures.” Fig. 3, A and B, refers to the labeling that occurred from incubations that contained exogenously added GlcNAC-P-P-dolichol. The material in Fig. 3A is derived from the GlcNAC region of the paper chromatogram and shows glucosaminol as the major labeled component. This would be the derivative formed from GlcNAC-P-P-dolichol synthesized de novo during the incubation. The relatively small amounts of labeled GlcNH$_2$ in Fig. 3A most likely are due to the presence of contaminating GlcNAC-GlcNAC that accompanied the GlcNAC region when cut and eluted from the paper chromatogram. The source of the labeled peak material in Fig. 3B is chitobiose recovered from the paper chromatogram showing the greatly enhanced labeling in GlcNH$_2$ derived from the non-reducing end of $[^3\text{H}]$GlcNAC-GlcNAC-P-P-dolichol formed by the addition of $[^3\text{H}]$GlcNAC to the exogenously added GlcNAC-P-P-dolichol. Fig. 3, C and D, indicates the labeling that occurred from incubations carried out under basal conditions, i.e. in the absence of exogenously added GlcNAC-P-P-dolichol. The material in Fig. 3C comes from processing the GlcNAC region of the paper chromatogram. As with Fig. 3A, labeled glucosaminol in Fig. 3C arises from de novo synthesized GlcNAC-P-P-dolichol and the small amount of GlcNH$_2$ from incompletely resolved chitobiose after paper chromatography. The material in Fig. 3D is derived from processing the material in the GlcNAC-GlcNAC region of the chromatogram. As seen in Fig. 3D, under basal conditions glucosamine derived from the non-reducing end of the chitobiosyl derivative and glucosaminol from the reducing terminus were labeled to a similar extent, consistent with both sugar residues originating directly from the same donor source, i.e. UDP-$[^3\text{H}]$GlcNAC, and the reaction approaching isotopic equilibrium under the conditions of this experiment. In contrast, in the presence of exogenously added GlcNAC-P-P-dolichol (Fig. 3B), no discernible peak was detected in the GlcNH$_2$-OH region as would be predicted if the exogenously added unlabeled GlcNAC-P-P-dolichol was the major, if not only, species converted to the chitobiosyl dolichol (18 $\mu$m). C, presence of $[^4\text{C}]$Man-P-dolichol (2.1 $\mu$m) and absence (●) or presence (○) of exogenously added GlcNAC-P-P-dolichol (18 $\mu$m). D, absence (●) or presence (○) of GlcNAC-GlcNAC-P-P-dolichol (18 $\mu$m). E, presence of Man-P-Dol (4.8 $\mu$m) and the absence (●) or presence (○) of GlcNAC-GlcNAC-P-P-dolichol (19 $\mu$m).
derivative. Thus, the experimental evidence supports the previous suggestion that the apparent lack of inhibition of formation of [3H]GlcNAc-P-P-dolichol that occurred in the presence of exogenously added GlcNAc-P-P-dolichol under basal conditions in the absence of showdomycin reflects the competition by the large excess of unlabeled exogenously added GlcNAc-P-P-dolichol to serve as a substrate for the synthesis of the chitobiosyl derivative. When the synthesis of the chitobiosyl derivative is inhibited as with the use of showdomycin, these additional influences are eliminated, and the inhibitory effect is directly seen.

The presence of detergent in the incubation mixtures argues against the possibility that the difference in response to showdomycin at the basal level was due to the presence of different pools of added versus newly synthesized GlcNAc-P-P-dolichol.

**Influence of Mild Acid Treatment of GlcNAc Lipids; Influence of Potential Hydrolytic Products, Other GlcNAc Lipids**

These studies have shown that exogenously added GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol inhibit the de novo synthesis of GlcNAc-P-P-dolichol. The effect of these compounds was examined after they were subjected to mild acid hydrolysis. This was performed in 0.1N HCl in the presence of tunicamycin (0.4 μg/ml) was blocked virtually 100% (data not shown).

This observation argues against contributions to the effects described by compounds such as GlcNAc-containing glycosphingolipids or GlcNAc/glucosaminylphosphatidylinositolides involved in glycosphingolipid anchor biosynthesis (34).

These control studies support the proposal that the inhibition observed were due specifically to the presence of the mono-GlcNAc and chitobiosyl-P-P-dolichol derivatives.

**Stability of GlcNAc-GlcNAc-P-P-dolichol during Incubation**

The possibility was examined that the inhibition of the formation of GlcNAc-P-P-dolichol by GlcNAc-GlcNAc-P-P-dolichol under these experimental conditions was due to the instability of the chitobiosyl derivative and its formation in situ of GlcNAc-P-P-dolichol which actually brought about the effect. Glc-
**Feedback and Product Inhibition of GlcNAc-P-P-Dol Biosynthesis**

Two substrates, dolichol phosphate and UDP-GlcNAc, participate in the formation of GlcNAc-P-P-dolichol. The steady state kinetics of this reaction in the presence of the two intermediates of the dolichol pathway which these studies have shown to inhibit its activity were examined, and the data are summarized in Fig. 4. As seen in Fig. 4A, in the presence of exogenous GlcNAc-P-P-dolichol added at 7.5- and 15-fold higher concentrations than that produced in the absence of the inhibitor, the data indicate that this inhibitor is competitive versus UDP-GlcNAc, i.e. UDP-GlcNAc and GlcNAc-P-P-dolichol compete for the same site or same form of the enzyme. In contrast, (Fig. 4B) non-competitive or mixed type inhibition was demonstrated versus dolichol phosphate. The kinetics of inhibition by GlcNAc-GlcNAc-P-P-dolichol are seen in Fig. 4, C and D. When examined as a function of variation in the concentration of dolichol phosphate, competitive inhibition is seen (Fig. 4D). Variation in the concentration of UDP-GlcNAc, however, revealed that GlcNAc-GlcNAc-P-P-dolichol is an uncompetitive inhibitor versus UDP-GlcNAc (Fig. 4C) where the apparent V<sub>max</sub> and apparent K<sub>m</sub> values are each changed to a similar extent, as indicated by the parallel lines. The inhibition constants obtained from the non-linear least squares analysis are tabulated in Table III.

**Stimulation by GlcNAc-P-P-dolichol of Man-P-Dol Biosynthesis**

In view of previous observations that Man-P-Dol can stimulate GlcNAc-P-P-dolichol formation (7–15), and the present observation that exogenously added GlcNAc-P-P-dolichol can inhibit its own formation even when stimulated by Man-P-dol, it was of interest to examine the converse situation, i.e. the effect of GlcNAc-P-P-dolichol on Man-P-Dol synthesis.

**Retina Microsomes—Incubation of retina microsomes under optimal conditions for Man-P-Dol formation, as described previously (24) in the presence of exogenously added GlcNAc-P-P-dolichol, resulted in an enhanced formation of Man-P-dol, as seen in Fig. 5A. From Lineweaver-Burk analyses of the data, apparent K<sub>m</sub> and V<sub>max</sub> values were calculated, as summarized in Table IV. Thin layer chromatography in solvent systems 1

---

Fig. 3. High voltage paper electrophoresis of glucosaminyl residues after incubation in the absence of showdomycin. Incubation mixtures, scaled up 4-fold over that described under “Experimental Procedures,” contained dolichol phosphate, Tes buffer, Mg<sup>2+</sup>, Triton X-100, UDP-[<sup>3</sup>H]GlcNAc, microsomes, and where indicated, GlcNAc-P-P-dolichol (18 μM). After solvent partitioning of the products, mild acid hydrolysis, separation of GlcNAc and GlcNAc-GlcNAc by paper chromatography, and recovery from the chromatogram, followed by reduction with NaBH<sub>4</sub> and strong acid hydrolysis (4 N HCl, 6 h), the products were examined by high voltage paper electrophoresis on Whatman 3MM paper in 1% sodium tetraborate for 60 min at 53 V/cm. The electrophoretogram was cut into 1 × 4-cm zones, and the radioactivity was measured by scintillation spectrometry. A, the pattern obtained after processing the GlcNAc region after paper chromatography of the products formed in the presence of GlcNAc-P-P-dolichol. B, the pattern obtained after processing the GlcNAc-GlcNAc region of the chromatogram formed in the presence of GlcNAc-P-P-dolichol. C, the same as A, i.e. the GlcNAc region, except that the incubation had been performed under basal conditions, i.e. in the absence of GlcNAc-P-P-dolichol. D, the same as B, i.e. the GlcNAc-GlcNAc region, except that the incubation was performed under basal conditions. The arrows indicate the mobilities of authentic [<sup>3</sup>H]GlcNH<sub>2</sub> and [<sup>3</sup>H]GlcNH<sub>2</sub>OH.
and 2 of the radioactive product formed under these conditions showed the presence of a single radioactive area that migrated with purified, standard Man-P-dolichol (data not shown).

**Yeast Enzyme**—The stimulation by GlcNAc-P-P-dolichol of Man-P-Dol synthesis was examined further using a purified, recombinant yeast Man-P-Dol synthase (25). After incubation under initial rate conditions, the same response was obtained as with the retina microsomes as shown in Fig. 5B. From Lineweaver-Burk analysis of the data, apparent $K_a$ and $V_{\text{max}}$ values were calculated, as summarized in Table IV. Thin layer chromatography in solvent systems 1 and 2 showed a single radioactive component migrating with standard Man-P-Dol (data not shown).

**DISCUSSION**

In all eukaryotic organisms thus far investigated, the biosynthesis of the core region oligosaccharide of asparagine-linked glycoproteins proceeds via the dolichol pathway. The following Reaction 1

\[
\text{Dolichol phosphate + UDP-GlcNAc} \rightarrow \text{GlcNAc-P-P-dolichol + UMP}
\]

has been described as the first committed step of this complex sequence of reactions (35). Regulation of the formation of GlcNAc-P-P-dolichol could thus clearly influence the steady state concentration of the final product of the pathway, Glc$_3$Man$_{39}$-...
GlcNAc-P-P-dolichol, required for glycoprotein biosynthesis. In accord with the sensitive site in metabolism that this reaction may occupy, down-regulation of GPT-1 has been shown to inhibit the glycosylation and secretion of proteins by Xenopus oocytes (36) and cause defects in the life cycle of yeast (37). It has also been suggested that the accumulation of the large lipid-linked oligosaccharide might also influence the formation of other earlier intermediates of the pathway, such as GlcNAc-P-P-dolichol, by feedback inhibition (38). Several influences of potential regulatory nature have been described concerning the activity of the GlcNAc-transferase that catalyzes the formation of GlcNAc-P-P-dolichol. Treatment of chick oviduct membranes with diethylstilbesterol was observed to increase its production (2). Allosteric stimulation of the synthesis of this compound by Man-P-Dol has been described (7–15). In this regard, it has been demonstrated (17, 18) that the equilibrium constant for this reaction has not should be readily reversible, as has been demonstrated (17, 18). The biosynthesis of GlcNAc-P-P-dolichol involving an exchange of pyrophosphate bonds on either side of the equation should be readily reversible, as has been demonstrated (17, 18). Although the equilibrium constant for this reaction has not
been reported, the results of the present studies have demonstrated that the inhibition of the formation of GlcNAc-P-P-dolichol, however, cannot be a function only of the concentration of the product driving the reaction backward by mass action. Rather, in addition to the latter effect (the quantitative contribution of which cannot be determined as yet), more subtle influences on the kinetics of this reaction are involved, as demonstrated in the present work.

A Kinetic Model for the Mechanism of UDP-GlcNAc:Dolichyl Phosphate N-Acetylg glucosamine 1-Phosphate Transferase (GPT-1)—Since patterns of product and substrate inhibition can provide a basis for analyzing the order of additions of substrate and products (40), the following model is suggested from the results of the kinetic studies for the mechanism of action of GPT-1. The steady state kinetic studies are all consistent with a sequential bi-bi mechanism with the feedback inhibition by GlcNAc-GlcNAc-P-P-dolichol being caused by the formation of a ternary dead-end complex with UDP-GlcNAc as shown in Scheme 2.

The data that strongly corroborate the ordered addition and release of substrates are the competitive inhibition of GlcNAc-P-P-dolichol with UDP-GlcNAc as the varied substrate. This indicates that the binding of the sugar nucleotide and GlcNAc-P-P-dolichol is a mutually exclusive event, i.e. they compete for the same form of the enzyme. The initial addition of UDP-GlcNAc is further confirmed by the uncompetitive inhibition pattern with GlcNAc-GlcNAc-P-P-dolichol as the inhibitor. Uncompetitive inhibition arises when the inhibitor binding occurs subsequent to the binding of the varied substrate (40, 41). The competitive pattern of GlcNAc-GlcNAc-P-P-dolichol versus dolichol phosphate indicates that the inhibitor and the substrate compete to bind to the E/UDP-GlcNAc binary complex. The non-competitive inhibition by GlcNAc-GlcNAc-P-P-dolichol versus dolichol phosphate is consistent with the mechanism in Scheme 2. This inhibition arises because GlcNAc-P-P-dolichol can bind to form an inhibitory complex whether dolichol phosphate is present at low or high concentrations. The magnitude of the Ki values (Table III) do not necessarily reflect only the affinity of GlcNAc-P-P-dolichol for a given enzyme form but are dependent on the fraction of the enzyme present in that form (40). It is anticipated that the specific Ki values determined in these studies will reflect a greater affinity, i.e. lower Ki than that determined in the activity assay (Table I) because the Ki and Ks terms reflect an extrapolation to substrate concentrations where a single enzyme form predominates, whereas with the activity assay employing substrates near their Km values several different enzyme forms will be present at steady state.

It might be argued that the data from 2.88 μM GlcNAc-P-P-dolichol in Fig. 4A would suggest non-competitive inhibition because drawing a line derived from an analysis of each individual data point rather than as an entire data set will not intersect at a common point on the y axis. If we were to have concluded that this inhibition was non-competitive, a non-statistically significant Ki of 5.8 ± 4.3 μM would have been obtained. The two characteristics of being greater than the largest inhibitor concentration employed coupled with the large standard error are the hallmarks of an unnecessary kinetic parameter, i.e. one that is not required to accommodate the data (32). Concerning the magnitude of the deviations of the data points from the computed values, these systematic errors are less than 1σ, i.e. the systematic error is within the standard error or random variability of the assay. This variability is reflected in the standard errors of the reported inhibition constants of 10–25%.

The comparative results with GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol indicate that the formation of GlcNAc-P-P-dolichol and the reciprocal dolichol pathway.

References

1. Hemmings, F. W. (1995) in Glycoproteins, G. N. Entrevel, J., Vliegenthart, J., F. K. P., and Schachter, H., ed. pp. 127–143, Elsevier Science Publishers B. V., Amsterdam

2. Hayes, G. R., and Lucas, J. J. (1983) J. Biol. Chem. 258, 15095–15100

3. LeGrand, P., Pretel, R., Kesselheim, J., te Hessen, S., and Kuruzin, M. A. (1995) Glycobiology 5, 633–642

4. Abeijon, C., and Hirschberg, C. B. (1990) J. Biol. Chem. 265, 14691–14695

5. Kean, E. L. (1991) J. Biol. Chem. 266, 942–948

6. Dan, N. D., Middleton, R. B., and Lehrman, M. A. (1996) J. Biol. Chem. 271, 30717–30724

7. Kean, E. L. (1982) J. Biol. Chem. 257, 7952–7964

8. Kean, E. L. (1983) Biochim. Biophys. Acta 730, 248–253

9. Kean, E. L. (1983) Biochim. Biophys. Acta 732, 488–490

10. Kean, E. L. (1985) J. Biol. Chem. 260, 12561–12571

11. Kean, E. L., and DeBrakeleer, D. J. (1986) Arch. Biochem. Biophys. 250,
Feedback and Product Inhibition of GlcNAc-P-P-Dol Biosynthesis

12. Kean, E. L., Rush, J. S., and Waechter, C. J. (1994) Biochemistry 33, 10508–10512
13. Kaushal, G. P., and Elbein, A. D. (1985) J. Biol. Chem. 260, 16303–16309
14. Shailubhai, K., Dong-Yu, B., Saxena, E. S., and Vijay, I. K. (1988) J. Biol. Chem. 263, 15964–15972
15. Carson, D. D., Farrar, J. D., Laidlaw, J., and Wright, D. A. (1990) J. Biol. Chem. 265, 2947–2955
16. Kean, E. L., and Niu, N. (1998) Glycoconj. J. 15, 11–17
17. Heifetz, A., Keenan, R. W., and Elbein, A. D. (1979) Biochemistry 18, 2186–2192
18. Harford, J. B., and Waechter, C. J. (1979) Arch. Biochem. Biophys. 197, 424–435
19. Kean, E. L., and Wei, Z. (1998) Glycoconj. J. 15, 405–414
20. Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509
21. Imperiali, B., and Zimmerman, J. W. (1990) Tetrahedron Lett. 31, 6485–6488
22. Kean, E. L. (1977) J. Supramol. Struct. 7, 381–395
23. Kean, E. L. (1977) J. Biol. Chem. 252, 5622–5629
24. Kean, E. L. (1977) Exp. Eye Res. 25, 405–417
25. Schutzbach, J. S., Zimmerman, J. W., and Farsee, W. T. (1993) J. Biol. Chem. 268, 24190–24196
26. Lucas, J. J., Waechter, C. J., and Lennarz, W. J. (1975) J. Biol. Chem. 250, 1992–2002
27. Prasad, A. V. K., Plantner, J. J., and Kean, E. L. (1992) Exp. Eye Res. 54, 913–920
28. Kean, E. L. (1992) Biochem. Cell Biol. 70, 413–421
29. Kean, E. L. (1996) Glycoconj. J. 13, 675–680
30. Reissig, J. L., Strominger, J. L., and LeLor, L. F. (1955) J. Biol. Chem. 217, 959–966
31. Leatherbarrow, R. J. (1992) GraFit Version 3.0, Erithacus Software Ltd., Staines, UK
32. Cleland, W. W. (1979) Methods Enzymol. 63, 103–138
33. Barshop, B. A., Wrenn, R. F., and Frieden, C. (1985) Anal. Biochem. 139, 134–145
34. Bangs, J. D., Andrews, N. W., Hart, G. W., and Englund, P. T. (1986) J. Cell Biol. 103, 255–263
35. Lehman, M. A. (1991) Glycobiology 1, 553–562
36. Kukuruzhinska, M. A., Apekin, V., Lankin, M. S., Hiltz, A., Rodriguez, A., Lin, C. C., Paz, M. A., and Oppenheim, F. G. (1994) Biochem. Biophys. Res. Commun. 198, 1248–1254
37. Kukuruzhinska, M. A., and Lennon, K. (1995) Biochim. Biophys. Acta 1247, 51–59
38. Pan, Y. T., and Elbein, A. D. (1990) Biochemistry 29, 8077–8084
39. Dan, N., and Lehman, M. A. (1997) J. Biol. Chem. 272, 14214–14219
40. Cleland, W. W. (1963) Biochim. Biophys. Acta 67, 173–187
41. Cleland, W. W. (1963) Biochim. Biophys. Acta 67, 188–196