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Kinetics of formation of GlcNAc-GlcNAc-P-P-dolichol by microsomes from the retina of the embryonic chick

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Little quantitative information is available concerning individual reactions of the dolichol pathway. We have investigated the kinetics of the GlcNAc-transferase that catalyzes the biosynthesis of GlcNAc-GlcNAc-P-P-dolichol using chemically synthesized GlcNAc-P-P-dolichol as the substrate. Using microsomal preparations from the retina of the embryonic chick as enzyme source, optimal incubation conditions of pH, metal ion and detergent concentrations were established, after which apparent kinetic constants (K_m and V_{max}) were determined under initial rate conditions for GlcNAc-P-P-dolichol and UDP-GlcNAc. These studies provide the first quantitative description of the kinetics of this reaction.

***Keywords*: dolichol pathway, GlcNAc-P-P-dolichol, GlcNAc-GlcNAc-P-P-dolichol, kinetics, retina**

***Abbreviations*: GlcNAc-P-P-dolichol, *N*-acetylglucosaminylpyrophosphoryldolichol; GlcNAc-GlcNAc-P-P-dolichol, *N*-acetylglucosaminyl-*N*-acetylglucosaminylpyrophosphoryldolichol; TX-100, triton X-100; Tes, 2-{[*tris*-(hydroxymethyl)-methyl]-amino}-ethanesulfonic acid.**

Introduction

Research over the past 25 years by many laboratories has revealed that the dolichol pathway is the means whereby the core region of asparagine-linked glycoproteins is assembled [1]. There is still little information available, however, concerning properties of the individual enzymatic reactions that comprise this complex series of events. We have directed our attention to the initial steps of the pathway, the biosynthesis of GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol which proceeds by the reaction sequence:

Dolichyl phosphate + UDP-GlcNAc → GlcNAc-P-P-dolichol + UMP

GlcNAc-P-P-dolichol + UDP-GlcNAc → GlcNAc-GlcNAc-P-P-dolichol + UDP

These reactions are catalyzed by two separate *N*-acetylglucosaminyl transferases acting sequentially: UDP-GlcNAc:dolichyl phosphate, *N*-acetylglucosamine-1-phosphate transferase (GPT-1) and UDP-GlcNAc:GlcNAc-P-P-dolichol, N-acetylglucosamine transferase (GT-2) – a process initially proposed by Leloir *et al.* [2]. A variety of reports have described enzyme systems that result in the formation of both GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol [2–15]. While information concerning the kinetic properties of GPT-1 has been published [12, 15–18], quantitative data has not been available concerning the kinetic properties of the GlcNAc-transferase catalyzing the second reaction, the formation of GlcNAc-GlcNAc-P-P-dolichol. Using chemically synthesized GlcNAc-P-P-dolichol as the substrate and microsomes from the retina of the embryonic chick as enzyme source, we have investigated kinetic properties of this reaction.

Materials and methods

Enzyme preparations

Microsomes were prepared from the retinas of the 15–16 day old embryonic chick as described previously [11].

Materials

GlcNAc-P-P-dolichol, synthesized chemically [19], was provided by Dr Barbara Imperiali, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA. UDP[6-^{3}H]GlcNAc (37 Ci mmol^{-1})

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was obtained from Dupont-New England Nuclear Corp. (Boston, MA). Tunicamycin and showdomycin were obtained from Sigma Chemical Co. (St Louis, MO). Amphomycin was a gift from Bristol Laboratories, (Syracuse, NY), and diumycin, a gift from E.R. Squibb and Sons, Inc. (Princeton, NJ)‡.

Enzymatic assay for the synthesis of GlcNAc-GlcNAc-P-P-dolichol

Assay mixtures contained GlcNAc-P-P-dolichol (as indicated),UDP-[^{3}H]GlcNAc (170 dpm pmol^{-1}) (as indicated), Tes buffer (0.2 M, pH 7.4), TX-100 (0.15%), 6.7 mM $MgCl_2$ and microsomes (0.023 mg protein) in a total volume of 0.15 ml. GlcNAc-P-P-dolichol in chloroform : methanol (2 : 1) was evaporated to dryness with nitrogen, vortexed vigorously with TX-100, after which the other components of the reaction mixture were added. The incubations at 37 °C were started with the addition of microsomes and stopped by the addition of chloroform : methanol (2 : 1 by vol). After solvent partitioning by the procedure of Folch *et al.* [20], the radioactivity in the washed lower phase after evaporation to dryness was determined by scintillation spectrometry in the presence of 0.5 ml water and 5 ml EcoLume scintillation cocktail (ICN, Costa Mesa, CA), as described previously [12].

Mild acid hydrolysis, resin treatment

The GlcNAc-lipids were subjected to mild acid hydrolysis by reacting with 0.1 N HCl in 80% tetrahydrofuran at 50 °C for 100 min, as described previously [21]. The hydrolysate was evaporated to dryness, redissolved in water and applied to a mixed bed ion exchange resin column composed of 0.5 ml each of Ag-2 × 8 (200–400 mesh) acetate, and Ag-50×8 (200–400 mesh)H^+. The column was eluted with 20 ml water, the eluate evaporated to dryness and the products examined by chromatography, as described below.

Chromatography

Dionex: HPAEC

The product obtained as described above was redissolved in water and filtered through a 0.45 μm nylon acrodisk-13 filter (Gelman Sciences, Ann Arbor, MI). The filtrate and washings were evaporated to dryness and redissolved in 0.05 ml water. To an aliquot of this material was added 5 nmol 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 (HPAEC) (Dionex Corp., Sunnyvale, CA). The mixture was injected onto a CarboPAc 1 column (4×250 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^{-1}. The elution of the standards was followed by pulsed amperometric detection (HPAEC-PAD) using pulse potentials and durations as described previously [22]. Radioactivity in the products of the reactions was measured by scintillation spectrometry of 0.5 ml fractions collected from the PAD cell.

Paper chromatography

Paper chromatography on Whatman No. 1 paper was also used to investigate the nature of the products of the reaction, using n-butanol : pyridine : water (6 : 4 : 3, by vol) as solvent system as described previously [14].

Thin layer chromatography

Thin layer chromatography was performed using 20 cm×20 cm glass plates pre-coated with a 0.25 mm layer of silica gel 60, without fluorescent indicator (E. Merck, Darmstadt). The following solvent systems were used: (1) chloroform : methanol : water (60 : 35 : 6, by vol); (2) chloroform : methanol : ammonium hydroxide : water (65 : 35 : 4 : 4, by vol).

Other procedures

Protein concentration was determined by a modification of the procedure of Lowry *et al.* [23]. The concentration of GlcNAc-P-P-dolichol was determined by phosphate analysis [24] after strong acid hydrolysis (4 N HCl, 6 h, 100 °C). Apparent K_m and V_{max} values were calculated from Lineweaver-Burk double reciprocal plots after analysis of the data by computer using the Kcat program (BioMetalics, Princeton, NJ). The migrations of standard GlcNAc and *N*-*N*′-diacetylchitobiose on paper chromatograms were detected by the aniline/diphenylamine reaction [25].

Results

Optimal incubation conditions

Metal, detergent requirements

An absolute requirement for metal ions in the conversion of GlcNAc-P-P-dol to GlcNAc-GlcNAc-P-P-dolichol could not be demonstrated, in accord with previous observations [8]. As seen in Figure 1, however, a slight enhancement in activity was observed at 6.7 mM $MgCl_2$ which was used in subsequent incubations. At higher concentrations, Mg^{2+} was inhibitory. Other metal chlorides, examined at 6.7 mM, were either without effect or were inhibitory, exhibiting the following relative activities (% of control performed in the absence of added metal ion): Ca^{2+}, 97%; Mn^{2+}, 92%; Co^{2+}, 77%; Fe^{3+}, 63%; Zn^{2+}, 0; Cu^{2+}, 0. At 6.7 mM, EDTA had little effect while at higher concentrations

‡ Showdomycin, amphomycin and diumycin are no longer available from these sources.

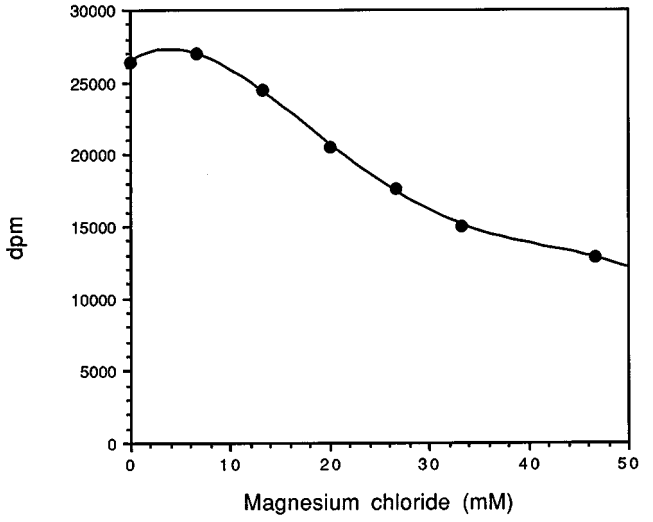

Figure 1. Variation in the concentration of $MgCl_2$. Incubations were performed for 15 min in the presence of GlcNAc-P-P-dolichol (20.3 μM), TX-100, UDP[^{3}H]GlcNAc 175 dpm mol^{-1}), Tes buffer, enzyme, and $MgCl_2$ as indicated on the abscissa, and GlcNAc-lipid formation assayed as described in Materials and methods.

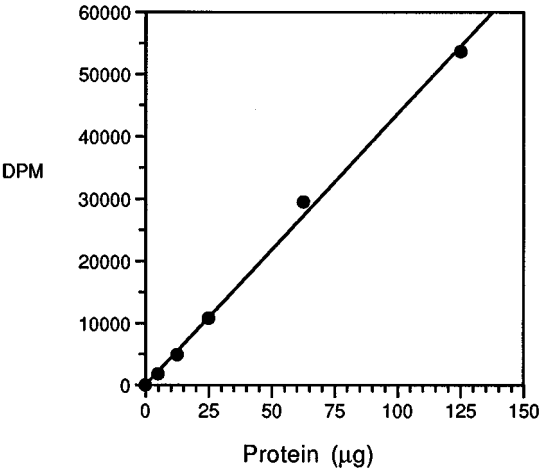

Figure 2. Variation in protein; effect on formation of $(GlcNAc)_2$-P-P-dolichol. Incubations were carried out for 15 min under the optimal conditions described in Materials and methods in the presence of GlcNAc-P-P-dolichol (20 μM), UDP[^{3}H]GlcNAc (201 μM, 87 dpm pmol^{-1}) and microsomes from the embryonic chick retina (protein as indicated) in a total volume of 0.15 ml. The incorporation of radioactivity into the products, indicated on ordinate, was measured as described in Materials and methods.

(47 mM), 29% inhibition was observed, unlike a previously reported stimulation in activity [8].

Detergent was required for these reactions in which exogenously added GlcNAc-P-P-dolichol was used as the substrate. Optimal activity was obtained using 0.15% TX-100 (data not shown).

Incubation time, protein concentration, pH optimum

At 37 °C the reaction was linear with time of incubation for at least 30 min (data not shown). Linearity with protein concentration was observed to about 130 μg protein, as seen in Figure. 2. This is in contrast to previous observations using dolichol phosphate as substrate, in which the formation of GlcNAc-lipids was not linear at low concentrations of protein [7, 12].

In the presence of 0.2 M Tes buffers the optimal pH was about 7.5 (Figure. 3).

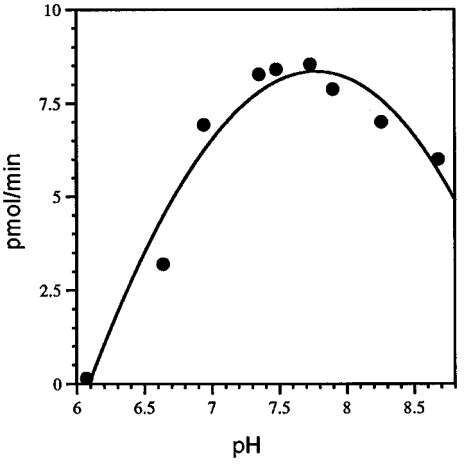

Figure 3. Effect of variation in pH on synthesis of GlcNAc-GlcNAc-P-P-dolichol. Incubations were carried out for 15 min as described in Materials and methods in the presence of GlcNAc-P-P-dolichol (10.1 μM), UDP[^{3}H]GlcNAc (50 μM, 160 dpm pmol^{-1}), enzyme (0.05 mg protein) and 0.2 M concentrations of Tes buffers whose pH is indicated on the abscissa.

Kinetic studies: variation in UDP-GlcNAc and GlcNAc-P-P-dolichol

When examined under optimal conditions as described above, saturation kinetics were observed upon variation of the concentrations of GlcNAc-P-P-dolichol (Figure 4) and of the sugar nucleotide, [^{3}H] UDP-GlcNAc (Figure 5). A summary of the kinetic data from such studies is seen in Table 1. The V_{max}/K_m ratio calculated from this data for the formation of the chitobiosyl derivative using GlcNAc-P-P-dolichol as substrate (Table 1) indicates a 550-fold enhanced efficiency of this reaction as compared to the value (0.25) previously obtained using dolichol phosphate as substrate for the formation of GlcNAc-lipids [12] in which a similar enzyme system was used. The increase in efficiency of this reaction calculated from variation in UDP-GlcNAc, in contrast, is only about five-fold over that obtained previously for GlcNAc-lipid synthesis using dolichol phosphate as substrate (8.2 vs 1.8).

Consistent with the inhibition seen with increasing concentrations of $MgCl_2$ (Figure 1), decreased efficiencies of the reactions were obtained at 27 mM $MgCl_2$ as compared to

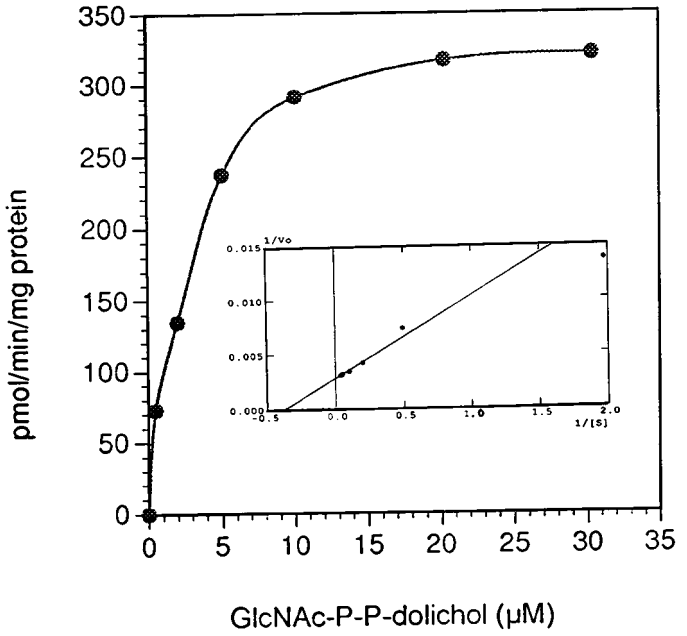

Figure 4. Effect of variation in [GlcNAc-P-P-dolichol] on enzymatic activity. Incubations were carried out for 15 min in the presence of 0.2 M Tes buffer, pH 7.5, $MgCl_2$ (6.7 mM), TX-100 (0.15%), UDP[^{3}H]GlcNAc (0.194 mM; 94 dpm pmol^{-1}), enzyme (0.022 mg protein) and GlcNAc-P-P-dolichol, as indicated on the abscissa, in a total volume of 0.15 ml. The data on the ordinate were calculated from the difference in radioactivity obtained in the absence of GlcNAc-P-P-dolichol (125 dpm) and that obtained in its presence using the procedures as described in Materials and methods. The radioactivity incorporated at 30 μM GlcNAc-P-P-dolichol, for example, was 10 300 dpm. In the inset is plot of 1/V vs 1/[S] from which kinetic data was calculated as given in Table 1.

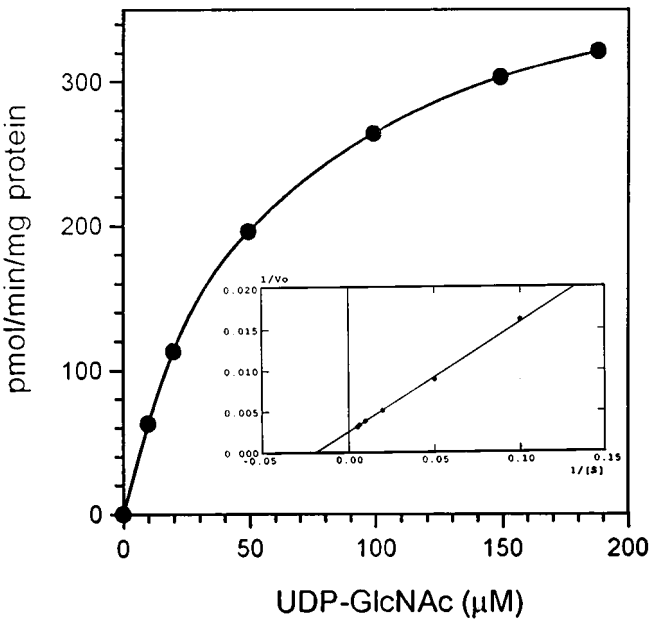

Figure 5. Effect of variation in [UDP-GlcNAc] on the biosynthesis of GlcNAc-GlcNAcs-P-P-dolichol. Incubations were performed in the same manner as described in the legend to Figure 4 in the presence of 25 μM GlcNAc-P-P-dolichol. The concentration of UDP-[^{3}H]-GlcNAc (84 dpm pmol^{-1}) was varied as indicated on the abscissa.

6.7 mM $MgCl_2$. Thus, comparing the V_{max}/K_m ratios for GlcNAc-P-P-dolichol and UDP-GlcNAc, the efficiencies of the reactions performed at the higher concentration of $MgCl_2$ were decreased four-fold and two-fold, respectively (data not shown).

Inhibitors

Compounds which have been shown to inhibit GlcNAc-transferase activities (see review [26]) were tested for their ability to inhibit the transfer of a residue of GlcNAc to the substrate, GlcNAc-P-P-dolichol. Concentrations were used that have been effective in other systems. As seen in Table 2, tunicamycin did not inhibit this reaction, consistent with other observations [14]. Amphomycin induced a partial inhibition, as did diumycin in accord with other observations [9, 27, 28]. The nucleoside antibiotic, showdomycin, essentially abolished activity. The D-(+) stereoisomer of showdomycin [29] showed the same inhibitory capacity as the material obtained from Sigma (data not shown). Extensive inhibition of GlcNAc-lipid synthesis was also obtained in the presence of the 2-deoxy derivative of showdomycin [30] (data not shown). These observations with the showdomycins are in contrast to their lack of or limited, ability to inhibit GlcNAc-lipid synthesis observed previously [31]. Inhibition by showdomycin and diumycin of the stimulation by mannosyl-P-dolichol of GlcNAc-lipid synthesis has been observed previously [32]. Nikkomycin had no effect on the transfer of GlcNAc to GlcNAc-P-P-dolichol at a concentration which inhibits the GlcNActransferases concerned with chitin formation [33].

Table 1. Kinetic properties of the GlcNActransferase catalyzing the biosynthesis of GlcNAc-GlcNAc-P-P-dolichol.

Substrate	*Apparent K_m (μM)*	*V_{max} (pmol min^{-1} mg protein^{-1})*	*V_{max}/K_m*
GlcNAc-P-P-dolichol	2.64±0.09(2)	363±5.0(2)	138±6.4
UDP-GlcNAc	57.1±2.9(2)	466±54(2)	8.15±0.53

Kinetic values were calculated by computer (using the K.cat program) from Linewear-Burk double reciprocal plots of data as seen in Figure 4 and 5. The values are presented as the mean±range of two separate determinations.

Other properties

The presence in the incubation mixtures of AMP (1.35 mM) and GDP (1.25 mM), shown previously to protect against

Table 2. Effect of various inhibitors of GlcNActransferases.

Inhibitor	*Concentration*	*dpm in products*	*% Inhibition*
None	-	11 200	0
Tunicamycin	0.02 mg ml^{-1}	12 200	0
Nikkomycin	5 μg ml^{-1}	11 400	0
Diumycin	0.05 mg ml^{-1}	6730	40
Amphomycin	0.67 mg ml^{-1}	4500	60
Showdomycin	0.67 mg ml^{-1}	175	98

Incubations were carried out for 20 min in the presence of GlcNAc-P-P-dolichol (19.5 μM), UDP[^{3}H]GlcNAc (104 μM, 164 dpm pmol^{-1}), enzyme (0.24 mg protein), and the other components of the incubation mixture as described in Materials and methods. Concentrations were used of inhibitors of GlcNActransferases shown to be effective in other studies. Enzymatic activity was followed by measuring the radioactivity present in the washed organic phase after the incubation mixture was partitioned by the Folch procedure, as described in Materials and methods.

the breakdown of UDP-GlcNAc in the formation of the chitobiosyl derivative by calf brain membranes [6], was without effect in the present studies (data not shown). The presence of sulfhydryl containing reagents also had little effect (data not shown).

Product identification

The major product seen after mild acid hydrolysis of the products formed using GlcNAc-P-P-dolichol as substrate had the same chromatographic properties when examined by Dionex HPAEC chromatography as *N*,*N*′diacetylchitobiose as seen in Figure 6B with trace amounts of the mono-GlcNAc derivative. For comparison, from incubations in which dolichyl phosphate (15.6 μM) was used as the substrate, both GlcNAc-lipids were formed (Figure 6A) as described previously [14].

Analysis by paper chromatography of the products of the reaction after mild acid hydrolysis revealed the presence of a single radioactive component which migrated in the same manner as standard *N*,*N*′diacetylchitobiose (data not shown).

Thin layer chromatography of the intact GlcNAc-lipids in two solvent systems (Figure 7, panels I and II) showed the presence of a single radioactive material (peak A, solid line) after incubation with GlcNAc-P-P-dol. This is in contrast to two peaks seen after incubation with dol-P (A and B, dashed line). The Rf values for peak A (0.21 (solid line) and 0.22 (dashed line)), and for peak B(0.31) using chloroform : methanol : water (60 : 35 : 6) in panel I are the same as described previously [8] for GlcNAc-GlcNAc-P-P-dol and GlcNAc-P-P-dol, respectively using this solvent system. The relative migration of these materials is also similar to that described previously [28] for these compounds synthesized by the retina.

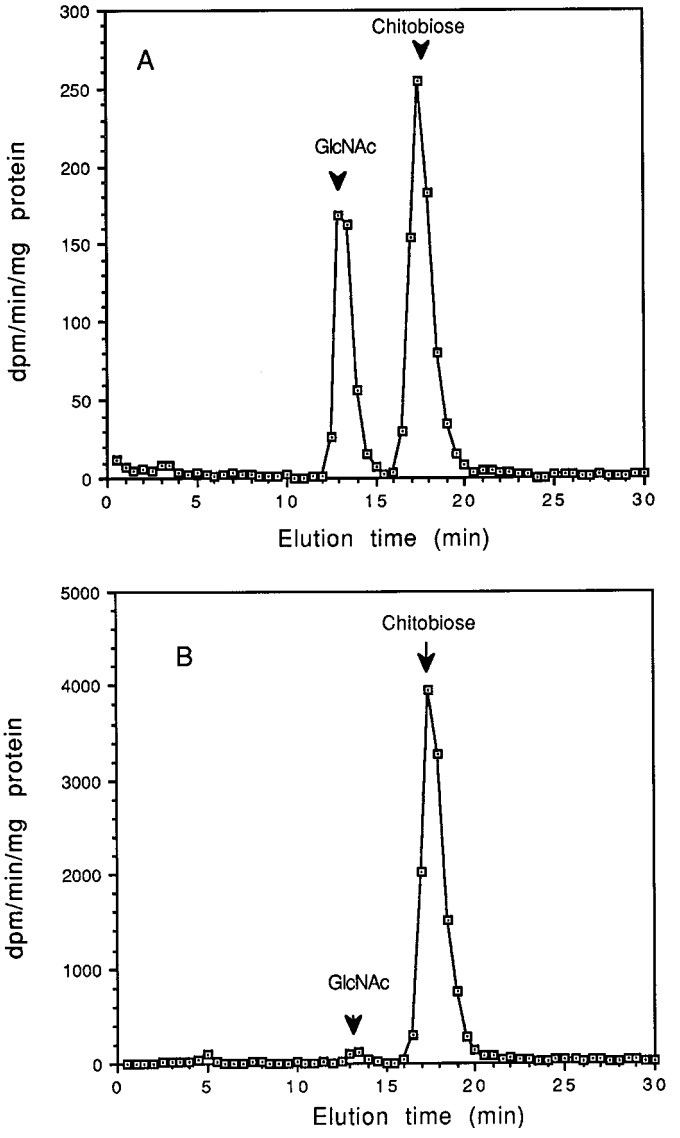

Figure 6. Analysis by Dionex chromatography of the products of GlcNAc-lipid synthesis. A. Large scale incubations were carried out for 15 min under optimal basal conditions of detergent, Mg^{2+} (27 μM), and Tes buffer (0.2 M, pH 7.4), as described previously [11, 12] using dolichyl phosphate as substrate (15.6 μM), UDP[^{3}H]-GlcNAc (47 μM, 177 dpm pmo^{-1}), and enzyme (1.2 mg protein ml^{-1}) in a total volume of 1.5 ml. B. Incubations were carried out as described in Materials and methods for 20 min in the presence of detergent, buffer, Mg^{2+}, UDP[^{3}H]GlcNAc (140 μM, 88 dpm pmol^{-1}), enzyme (0.092 mg protein) using GlcNAc-P-P-dolichol as substrate (39 μM) in a total volume of 0.6 ml. Analyses were performed as described in Materials and methods. The arrows indicate the elution times of GlcNAc and chitobiose present as internal standards, while the ordinate refers to radioactivity present in the fractions collected from the PAD cell.

Thus, although the lipoidal component has not been identified, the evidence obtained using different chromatographic systems, supports the conclusion that the product of the incubations using GlcNAc-P-P-dolichol as substrate is GlcNAc-GlcNAc-P-P-dolichol.

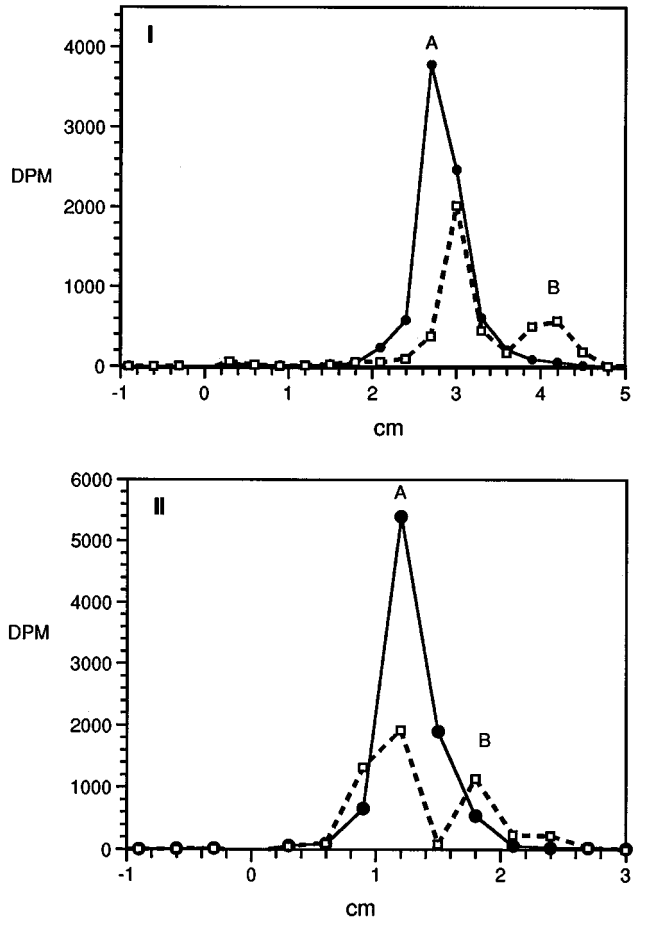

Figure 7. TLC of GlcNAc-lipids. Incubations were carried out in the presence of UDP[^{3}H]GlcNAc, TX-100, $MgCl_2$, Tes buffer, and enzyme as described in Materials and methods and in the legend to Figure 6, using dol-P (--□--) or GlcNAc-P-Pdol (—●—) as substrates. The products, after extraction by the Folch procedure [20], were examined by TLC on silica gel 60 plates (0.25 mm) using chloroform : methanol : water (60 : 35 : 6 by vol) as solvent system (panel I), or chloroform : methanol : NH_4OH : water (65 : 35 : 4 : 4 by vol) (panel II). The radioactivity in 0.3 cm×1.5 cm zones scraped from each plate was determined by scintillation spectrometry. (A) GlcNAc-GlcNAc-P-P-dolichol; (B) GlcNAc-P-P-dolichol.

Concluding remarks

Although a variety of reports have described the formation of GlcNAc-GlcNAc-P-P-dolichol, and an extensive characterization of the biosynthesis of this compound has been reported [8], this is the first quantitative description of the kinetics of the *N*-acetylglucosaminyl-transferase catalysing the addition of the GlcNAc residue to GlcNAc-P-P-dolichol.

Preliminary evidence has recently been presented [34] indicating that the chitobiosyl derivative, in addition to being an intermediate in the assembly of the core region oligosaccharide, may also have a regulatory function [Kean *et al.* manuscript in preparation]. Feed-back inhibition by GlcNAc-GlcNAc-P-P-dolichol of the formation of GlcNAc-P-P-dolichol was demonstrated. This property was also expressed when the reaction was stimulated by mannosyl-P-dolichol. Previous studies on regulation of the reactions of the dolichol pathway have revealed that mannosyl-P-dolichol acts as an activator of the GlcNActransferase concerned with the formation of GlcNAc-P-P-dolichol [11–16, 18, 35, 36], but not of the enzyme that adds the second GlcNAc residue [12, 14]. The present studies, describing some of the kinetics of the formation of the chitobiosyl derivative will aid in further investigations concerning regulation of these early reactions of the dolichol pathway.

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