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Dolichylpyrophosphate Oligosaccharides: Large-Scale Isolation and Evaluation as Oligosaccharyltransferase Substrates

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Abstract—Oligosaccharyltransferase (OST) catalyzes the transfer of a branched oligosaccharide from a dolichylpyrophosphate oligosaccharide (Dol-PP-OS) to the asparagine of a nascent polypeptide chain in vivo and peptide substrates in vitro. Here we report the isolation and purification of Dol-PP-OS from bovine pancreas and thyroid. Steady-state kinetic parameters comparing the two Dol-PP-OS to a shorter dolichylpyrophosphate disaccharide (DolPP-DS) previously synthesized in our laboratory are reported. These were determined for Dol-PP-OS, Dol-PP-DS, and the tripeptide Bz-Asn-Leu-Thr-NH_2 with solubilized OST and, for the first time, saturation kinetics were observed for all substrates. The kinetic data provide a basis for analyzing quantitatively the individual contributions of oligosaccharide donor and peptide acceptor substrates to OST-catalyzed glycosylation.

Introduction

N-Linked glycosylation is a common type of protein modification in which complex oligosaccharides are attached to specific asparagine residues.[1–3] This cotranslational process occurs in the rough endoplasmic reticulum and is catalyzed by the enzyme oligosaccharyltransferase (OST, EC 2.4.1.119).[4–7] The glycosylation process catalyzed by OST occurs on the nascent polypeptide chain during translocation from the ribosome to the lumenal face of the rough endoplasmic reticulum.

A sequon of three amino acids, Asn-X-Thr/Ser, where X is any amino acid except for proline, is necessary but not sufficient for glycosylation.[8,9] The tripeptide sequon itself is able to serve as a substrate for OST when both the amino and carboxy termini are blocked.[10] For the lipidoligosaccharide there is a strict requirement for a large polyisoprene (e.g. dolichol) as the lipid moiety.[11] The in vivo lipidoligosaccharide substrate for OST, dolichylpyrophosphate oligosaccharide (Dol-PP-OS), contains a complex branched oligosaccharide consisting of $Glc_3Man_9(GlcNAc)_2$. However, OST will utilize substrates with truncated oligosaccharide moieties. The minimal substrate that the enzyme recognizes is Dol-PP-$(GlcNAc)_2$ (dolichylpyrophosphate disaccharide, DolPPDS) whereas the monosaccharide, Dol-PP-GlcNAc is not a substrate for OST.[12] Due to the structural complexity of the lipidoligosaccharide, kinetic and mechanistic studies of OST have used either Dol-PP-OS that can be produced biosynthetically in very small amounts[12–16] or Dol-PP-DS which can be synthesized in larger quantities.[17–21] In this study, Dol-PP-OS was isolated from bovine pancreas and thyroid in mg quantities using modifications of published procedures.[22–25] This isolation and purification of Dol-PP-OS from bovine tissue marks the first time that sufficient quantities of this substrate have been available for a complete kinetic evaluation of the OST-catalyzed reaction. As a result, the steady-state kinetic parameters for OST-catalyzed glycosylation of the tripeptide, Bz-Asn-Leu-Thr-NH_2, by either bovine Dol-PP-OS or synthetic Dol-PP-DS can be determined. The data provide, under conditions where saturation can be observed for all substrates, the most complete kinetic analysis to date of the oligosaccharide donor substrate for this key step in *N*-linked glycoprotein biosynthesis[26] and set the stage for future use of these donors in more extensive kinetic studies.

Experimental

Materials

GDP-1-[^{3}H]-mannose and 7-[^{14}C]-benzoic acid were purchased from American Radiolabeled Chemicals (St. Louis). Mature bovine pancreas and thyroid were obtained from Pel-Freeze Biologicals. DE-52 cellulose

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was from Whatman, concanavalin A (Con A)-Sepharose from Sigma, and BioGel P-4 and P-6 from BioRad. P40 yeast microsomes and biosynthetic [^{3}H]-Dol-PP-OS were prepared as described by Clark et al.[16] Dol-PP-DS[11,19] was a gift of Mr. Xinggao Fang of this laboratory. Purified biosynthetic [^{14}C]$Glc_3Man_9(GlcNAc)_2$ and [^{14}C]$Man_9(GlcNAc)_2$ were generously provided by Dr. Robert Trimble, New York Department of Health, Albany, New York. All other reagents were of the highest grade available.

Isolation of unlabeled Dol-PP-OS from bovine tissue

Dol-PP-OS was isolated from bovine pancreas or thyroid by modification of the procedure of Spiro et al.[23] Frozen bovine pancreas or thyroid (240–300 g) were thawed in 1000–1500 mL of homogenization buffer (0.15 M Tris, pH 7.4, 4 mM $MgCl_2$) for 1 h at 4 °C. The tissue was homogenized in a blender for a total of 5 min in pulses of 1 min on/off at 4 °C. The homogenate (40 mL) was extracted with 175 mL $CHCl_3$:CH_3OH (3:2, v/v) via constant shaking at room temperature for 10 min. The mixture was centrifuged at 500 g for 15 min at 10 °C and the upper and lower phases were carefully removed by aspiration leaving the solid interface material. The interface material was collected and when all the homogenate had been extracted, the solid material was divided equally between six Teflon centrifuge bottles. The interface material was extracted three more times with $CHCl_3$:CH_3OH:H_2O (3:2:1, v/v/v, 100 mL/bottle); the aqueous component of the first extraction solvent contained 4 mM $MgCl_2$. After each addition of solvent, the samples were kept at room temperature for 10 min with constant shaking and then centrifuged at 500 *g* for 15 min at 10 °C. After each extraction the upper and lower phases were carefully removed from the solid material at the interface by aspiration. The interface material was then placed under vacuum for 2 h.

The partially dried material was resuspended in $CHCl_3$:CH_3OH (2:1, v/v; 720 mL total) and kept at room temperature with constant shaking for 10–20 min. Following centrifugation (as described above) the supernatant was carefully removed by aspiration. After repeating the 2:1 wash, Dol-PP-OS was extracted from the pellet by the addition of $CHCl_3$:CH_3OH:H_2O (1:1:0.3, v/v/v; 720 mL total) and shaking constantly at room temperature for 10–20 min. The supernatant was isolated by centrifugation and carefully removed to a clean flask. The pellet was extracted two more times and the extracts pooled. The organic solvents were removed by rotary evaporation at 30 °C and the remaining water was removed by lyophilization. The sample was stored at −80 °C.

Purification of bovine Dol-PP-OS

Dol-PP-OS from bovine pancreas or thyroid was purified by chromatography on DEAE cellulose (DE52, 4.8×30 cm Kontes Chromaflex column) employing a stepwise washing and elution followed by lectin affinity chromatography using Con A-Sepharose.[25] The DE52 column (acetate form, 150 mL) was equilibrated with 1.5 L CH_3OH, and 1.5 L $CHCl_3$:CH_3OH:H_2O (1:1:0.3, v/v/v). The isolated bovine Dol-PP-OS obtained from 1–3 extractions was dissolved in 50–100 mL of $CHCl_3$:CH_3OH:H_2O (1:1:1, v/v/v) and [^{3}H]-Dol-P-P-OS (110 000–132 000 dpm) was added as a marker. The sample was loaded onto the column and the column was washed successively with 150 mL $CHCl_3$:CH_3OH:H_2O (1:1:0.3, v/v/v), 150 mL CH_3OH, 1.5 L $CHCl_3$:CH_3OH (2:1, v/v), and 1.5 L $CHCl_3$:CH_3OH (2:1, v/v) containing 30 mM ammonium acetate. Dol-PP-OS was then eluted from the column as a sharp peak with $CHCl_3$:CH_3OH:H_2O (1:1:0.3, v/v/v) containing 0.15 M ammonium acetate. Fractions (~10–13 mL) were collected and the column monitored by absorbance at 260 nm. Aliquots (0.1 mL) were removed and analyzed by scintillation counting. The fractions containing Dol-PP-OS were pooled, the organic solvents were removed by rotary evaporation, and the water removed by lyophilization. The sample was dissolved in water and lyophilized several times to remove residual ammonium acetate.

Dol-PP-OS isolated by DEAE ion exchange chromatography was further purified by chromatography on a Con A-Sepharose lectin affinity column. A column of Con A-Sepharose (15 mL) was washed with 5–10 column volumes of equilibrating buffer (1% nonidet P-40 (NP-40), 0.1 M NaCl, 0.01 M Tris, pH 7.5, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$). The sample of DEAE-purified Dol-PP-OS was dissolved in 0.5–0.7 column volumes of equilibrating buffer and applied to the column. The sample was loaded onto the column, the flow was stopped, and the column and sample were allowed to equilibrate for at least 1 h at 4 °C. The column was then washed with equilibrating buffer and 3–5 mL fractions were collected. The wash was monitored by absorbance at 295 nm and 50 μL aliquots were removed for scintillation counting. When both the absorbance and radioactivity had returned to baseline values (approximately 3 column volumes), 0.7–0.8 column volumes of 8% α-methylmannoside in equilibrating buffer was applied to the column. The flow was stopped and the column was allowed to equilibrate at room temperature for 1 h, after which the Dol-PP-OS was eluted from the column with additional α-methylmannoside buffer. Fractions were collected and analyzed by absorbance at 295 nm and 50 μL aliquots were analyzed by scintillation counting. The eluent fractions containing tritium were pooled and lyophilized.

To remove α-methylmannoside, the residue from the Con A column was dissolved in 10–20 mL of 1% NP-40 and loaded onto a Bio-Gel P-6 column (1.5×35 cm) previously equilibrated in 1% NP-40. Fractions (1 mL) were collected and radioactivity determined on 50 μL aliquots. α-Methylmannoside elution was monitored by the phenol–H_2SO_4 reaction[27] carried out on 2 μL aliquots and absorbance was measured at 490 nm. Fractions containing Dol-PP-OS were pooled and lyophilized.

To remove the NP-40 detergent the sample was dissolved in $CHCl_3$:CH_3OH:H_2O (1:1:0.3, v/v/v) and applied to a DE52 column (1.0×5.0 cm) equilibrated with CH_3OH followed by $CHCl_3$:CH_3OH:H_2O (1:1:0.3,

v/v/v). The column was washed with $CHCl_3$:CH_3OH:H_2O (1:1:0.3, v/v/v) and the Dol-PP-OS eluted with $CHCl_3$:CH_3OH:H_2O (1:1:1, v/v/v) containing 0.15 M ammonium acetate. Fractions were collected and radioactivity determined on 50 μL aliquots. The radioactive fractions were pooled, concentrated by rotary evaporation, and lyophilized. Ammonium acetate was removed by repeated washings with water followed by lyophilization. The final yield of Dol-PP-OS was 1.75 nmol/g tissue. The sample was stored at −80 °C.

The concentration of bovine Dol-PP-OS (pancreas and thyroid) was calculated by determining the concentration of *N*-acetylglucosamine (GlcNAc) in the sample, assuming that each mole of Dol-PP-OS contains two moles of GlcNAc. The GlcNAc concentration was determined either by GC/MS[28] at the Complex Carbohydrate Research Center, University of Georgia, or by high-pH anion-exchange chromatography.[29] Heterogeneity in the oligosaccharide moiety of Dol-PP-OS (pancreas and thyroid) was determined by mild acid hydrolysis and analysis by gel permeation chromatography using BioGel P-4 as described previously.[16]

Synthesis of N^{α}-[^{14}C]-benzoyl-Asn-Leu-Thr-NH_2

Benzoylation of the tripeptide, *H*-Asn-Leu-Thr-NH_2[16] with 7-[^{14}C]-benzoic acid was effected by EDC-mediated coupling. An aliquot of 7-[^{14}C]-benzoic acid (1.25 mL, 2.2 μmol, 56 mCi/mmol) in toluene was transferred to a reaction flask and the toluene was removed under a gentle stream of nitrogen. The residue was dissolved in 2 mL dry DMF and unlabeled benzoic acid (32 mg, 0.26 mmol), HOBt (36 mg, 0.26 mmol) and EDC (52 mg, 0.26 mmol) were added. The tripeptide, dissolved in 0.5 mL of dry DMF and NMM (60 μL, 0.55 mmol), was added dropwise to the reaction flask with constant stirring. The reaction was allowed to proceed with constant stirring overnight under nitrogen. After 15 h, the reaction was checked by TLC and no starting materials remained. A new spot which appeared at $R_f = 0.52$ was radioactive. The solvent was removed in vacuo and the residue was washed with EtOAc. The insoluble desired product was isolated by filtration, washed several times with EtOAc, then ether and finally dried under vacuum. This material was dissolved in a minimal volume of MeOH (~5 mL) and ether was added until an amorphous solid appeared. After remaining at 0 °C overnight, the solid was isolated by filtration, washed several times with ether, and dried under vacuum. The final yield was 54.1 mg (44.4%) and was homogeneous by TLC ($R_f = 0.52$, silica gel, BuOH:HAc:H_2O, 4:1:5, v/v/v, upper phase). The specific radioactivity, based on the weight of the product, was 0.35 μCi/μmol.

Solubilized P40 OST assay

P40 microsomes were solubilized by a modification of the method described by Chalifour and Spiro.[30] Microsomes were diluted 1:1 (v/v) into a buffer containing 0.05 M Tris, pH 7.5, 1.7% NP-40, 5 mM $MgCl_2$, and 5 mM $MnCl_2$ at 4 °C. After 10 strokes using a Dounce homogenizer, the solubilized P40 was isolated by ultracentrifugation at 158 000 g for 1 h. The supernatant was carefully removed and used immediately in the assay or frozen in liquid N_2 and stored at −70 °C for up to 5 weeks. The same preparation of solubilized OST was used for all assays.

The assay mixture contained 0.05 M Tris, pH 7.5, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 0.2% Triton X-100, 1 mM egg phosphatidylcholine, 0.4% NP-40, 5% DMSO and solubilized P40 microsomes (3.7–4.0 mg/mL) in a total volume of 100 μL. For assays containing Dol-PP-OS, either N^{α}-[^{14}C]-Bz-Asn-Leu-Thr-NH_2 was kept constant at 682 μM and Dol-PP-OS was varied from 0–236 μM or Dol-PP-OS was kept constant at 118 μM and 50 μM, for the pancreatic and thyroid tissue, respectively, and the tripeptide was varied from 0–655 μM. For assays with Dol-PP-DS, either N^{α}-[^{14}C]-Bz-Asn-Leu-Thr-NH_2 was constant at 1250 μM and Dol-PP-DS was varied from 0–990 μM or Dol-PP-DS was constant at 330 μM and the tripeptide was varied from 0–1250 μM. The assay mixture was kept at room temperature for 2 h with constant shaking at 250 rpm. The reaction was stopped by the addition of 3 mL of $CHCl_3$:CH_3OH (3:2, v/v). After incubating on ice for 30–60 min, the product was recovered by centrifugation at 1000 *g* for 15 min. The supernatant was removed, extracted with 1 mL of 4 mM $MgCl_2$, and the layers separated by centrifugation. The top aqueous layer was carefully removed and taken to dryness under vacuum (Savant Speed-Vac). The pellet obtained from centrifugation of the original quenched assay was washed with 3 mL of water, centrifuged and the supernatant taken to dryness under vacuum. Both of the dried samples were analyzed by RP-HPLC (see below). This procedure is summarized in Figure 1.

HPLC analysis of glycopeptide product

HPLC analysis of peptide substrates and glycopeptide products was performed on either an Altex or Waters liquid chromatography system (Vydac or Rainin C_{18} column, 4.6 mm × 25 cm, 5 μm). Samples taken to dryness were resuspended in water containing 0.1% TFA. The pH of each sample was checked and adjusted to pH < 7 if necessary. The mobile phase was 0.1% TFA in H_2O and the glycopeptide product was isolated utilizing a gradient of 10–30% acetonitrile over 20 min. Fractions (0.5 mL) were collected and analyzed for ^{14}C by scintillation counting. Routinely >90% of the radioactivity applied to the column was recovered in the various fractions collected. The amount of glycopeptide formed was calculated from the radioactivity recovered using a specific radioactivity of 0.35 μCi/μmol for the labeled peptide. The kinetic data were analyzed using the procedure of Lee and Wilson[31] and fit to the kinetic programs of Cleland[32] using programs adapted for use on the Macintosh microcomputer (KinetAsyst, Intellikinetics, Princeton, NJ).

Glycopeptide product inhibition studies

The [^{14}C]-glycopeptide product was isolated by RP-HPLC and was purified to homogeneity. Product

inhibition was determined using solubilized OST and the assay mixture contained approximately 5 nM of biosynthetic [^{3}H]-Dol-PP-OS, 180 μM of Bz-Asn-Leu-Thr-NH_2, solubilized OST (2.0–3.0 mg/mL) and 0–2.3 μM of [^{14}C]-glycopeptide in 100 μL. The assay was conducted and worked up as described above.

Results

Isolation and characterization of Dol-PP-OS

Dol-PP-OS was isolated from bovine pancreas and thyroid by extraction with chloroform:methanol:water, followed by chromatography on DEAE and Con A lectin affinity columns. This protocol yielded considerably larger amounts (mg) of Dol-PP-OS than that had been previously obtained (pg to ng) by biosynthesis.[12,16] The approximate yield from four individual isolations and purifications was 1.75 nmol/g tissue resulting in almost 5 mg of Dol-PP-OS. These results are comparable to those reported previously in the literature; 1.0 nmol/g tissue for thyroid[23] and 0.7 nmol/g tissue for pancreas.[25] Biosynthetically produced [^{3}H]-Dol-PP-OS was added as an internal standard prior to the purification of Dol-PP-OS from pancreas or thyroid and a 50–60% yield of the radioactivity was recovered in the final product.

Saccharide analysis of Dol-PP-OS from both thyroid and pancreas revealed a heterogeneous mixture of oligosaccharides. The addition of glycosidase inhibitors such as castanospermine and deoxynojirimycin to the extraction and chromatography buffers was without effect. It is possible that the freshly isolated tissue was not frozen rapidly enough by the commercial vendor (Pel-Freeze) to prevent partial hydrolytic degradation of the oligosaccharide prior to storage. Gel filtration chromatography (BioGel P4) of the oligosaccharide after acid hydrolysis from the lipid showed multiple species for pancreatic Dol-PP-OS and only three major forms from thyroid (data not shown). The oligosaccharides obtained from pancreatic Dol-PP-OS were truncated forms of the full oligosaccharide of $Glc_3Man_9(GlcNAc)_2$ with the predominate species estimated to be $Man_{5-6}(GlcNAc)_2$. Carbohydrate analysis confirmed the gel filtration data with a maximum ratio of 3:1 Man to GlcNAc. Gel filtration analysis also shows that there is a significant amount of free mannose present, suggesting that the ratio of Man to GlcNAc in the oligosaccharide hydrolysate is actually lower than 3:1. Dol-PP-OS from thyroid had only three species of oligosaccharide with $Glc_3Man_9(GlcNAc)_2$ and Man_9-$(GlcNAc)_2$ predominating. Carbohydrate analysis of the thyroid Dol-PP-OS showed a ratio of 4.6:1 of Man to GlcNAc suggesting that the oligosaccharide portion of thyroid Dol-PP-OS consists primarily of $Glc_3Man_9(GlcNAc)_2$ and Man_9-$(GlcNAc)_2$. This conclusion is supported by co-elution (BioGel P4) of the two major oligosaccharides derived from thyroid Dol-PP-OS with authentic [^{14}C]Glc_3Man_9-$(GlcNAc)_2$ and [^{14}C]$Man_9(GlcNAc)_2$ (data not shown).

OST assay with bovine Dol-PP-OS

Previous studies utilizing biosynthetic [^{3}H]-Dol-P-P-OS or -DS had established roughly which substrates and products were found in which extraction layer in the assay work up.[16,19] In the present work, the use of [^{14}C]-Bz-Asn-Leu-Thr-NH_2, combined with the higher levels of Dol-PP-OS and HPLC analysis of the various fractions, allowed for a more detailed analysis of the product distribution (Fig. 1). When Dol-PP-OS or Dol-PP-DS was the lipidoligosaccharide substrate with solubilized OST, a majority of the glycopeptide product was found in the supernatant after the $CHCl_3$:CH_3OH (3:2, v/v) extraction. Both final fractions containing product were pooled before HPLC analysis. An alternative to this method of product analysis involves isolation of product by binding to an immobilized lectin such as Con A.[24,33] However, this method was found to be more time consuming and less reproducible.

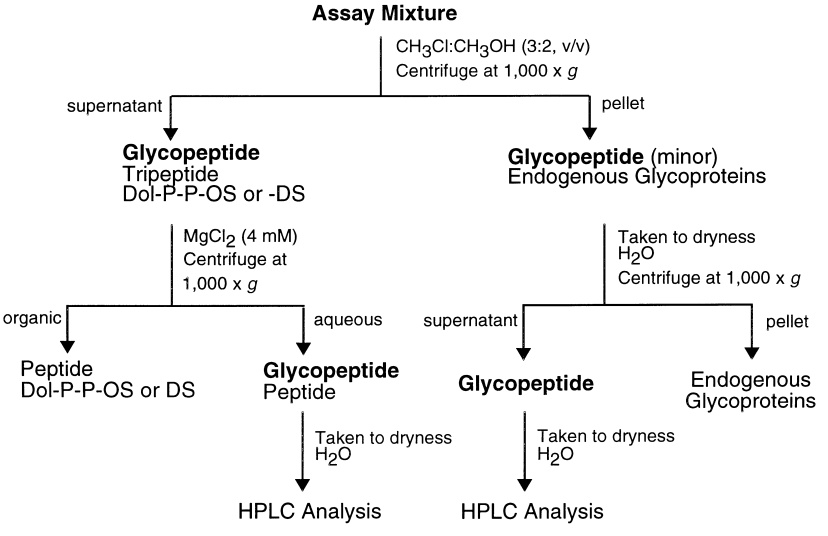

Figure 1. Isolation of the [^{14}C]-glycopeptide product. See text for details.

Kinetic constants for solubilized OST

To obtain the glycopeptide in product amounts that could be detected with good reproducibility, it was necessary to employ longer assay times and to accumulate product in concentrations greater than 10% of the initial substrate concentration. As a result, the kinetic parameters were determined using a modified Michaelis–Menton equation that takes into account the high concentration of product.[31] The amount of product formed was linear with respect to time for at least 2 h (data not shown). Possible product inhibition was addressed by isolating the glycopeptide product by HPLC and adding it to the OST assay. The concentration of glycopeptide ranged from 0.0 to 2.3 μM and the Dol-PP-OS was held constant at 0.005 μM. No product inhibition was observed under these conditions with a 600-fold excess of glycopeptide over substrate. Both the linearity of the assay with time and the lack of product inhibition allow for longer assay times and the accumulation of sufficient quantities of product to allow an accurate determination of the steady-state parameters. There was concern that substrate depletion would occur at the lower concentrations. Although this was not a problem with a 2-h assay, substrate depletion was observed at lower concentrations with a 3-h assay.

The velocity versus substrate concentration curves for pancreatic Dol-PP-OS and tripeptide with solubilized OST are shown in Figure 2. In both cases, saturating substrate levels were achieved. The kinetic constants derived from these data and comparable data from three other sets of substrates are given in Table 1. Similar data were obtained for microsomal membrane-bound OST (not shown). There was only a ca. twofold difference in the K_m between pancreatic Dol-PP-OS and Dol-PP-DS and a similar difference in K_m values of the acceptor peptide when these two donor substrates were used. K_m for the peptide decreased up to eightfold with an increase in the complexity of the oligosaccharide moiety of the lipidoligosaccharide substrate. V_{max} was similar under all conditions. The lower K_m for pancreatic Dol-PP-OS compared to Dol-PP-DS suggests that the K_m for thyroid Dol-PP-OS, containing a more complete complement of carbohydrates (vide supra), would be similar to or even lower than that for the pancreatic Dol-PP-OS.

Discussion

Prior to the research described herein, kinetic studies of the OST-catalyzed reaction using the natural oligosaccharide donors have been limited by the amount of Dol-PP-OS that can be produced biosynthetically. Significant quantities of Dol-PP-DS can be obtained synthetically[19,34,35] and have been utilized to study the mechanism of OST catalysis.[18,20,21,36] In the present study, Dol-PP-OS was isolated in mg quantities using modifications of previously published protocols[23,25] and the steady-state kinetic constants determined for the tripeptide, Bz-Asn-Leu-Thr-NH_2, Dol-PP-OS and Dol-PP-DS with solubilized OST. The OST-catalyzed reaction has generally been analyzed by selectively extracting the [^{3}H]-glycopeptide product from the biosynthetic [^{3}H]-lipidoligosaccharide substrate.[16,30] In the present work with bovine Dol-PP-OS and synthetic Dol-PP-DS, a radiolabelled peptide, [^{14}C]-Bz-Asn-Leu-Thr-NH_2, was the second substrate. The extraction schemes previously reported from this laboratory[16,20] were not adequate to completely separate the glycopeptide product from the peptide substrate. Separation by HPLC and analysis of the various fractions allowed for isolation of pure glycopeptide product (Fig. 1).

Dol-PP-OS is a slightly better substrate compared to Dol-PP-DS with solubilized OST. The values of K_m reported here (Table 1) represent an average for the various Dol-PP-OS species that are present since the sample is heterogeneous with respect to oligosaccharide and it is evident that this moiety plays an important role in substrate binding. It is assumed that OST recognizes all of the species; it has been reported that $Man_9(GlcNAc)_2$-PP-Dol is either not a substrate[12] or a poor substrate.[37] However, Dol-PP-OS from pancreas lacks any glucose and is efficiently transferred to a peptide substrate in situ suggesting that it is recognized by the enzyme.[38] Carbohydrate analysis of oligosaccharides derived from Dol-PP-OS isolated from both bovine pancreas and thyroid were predominantly $Man_{5-6}(GlcNAc)_2$ and $(Glc_3)Man_9(GlcNAc)_2$, respectively. It is

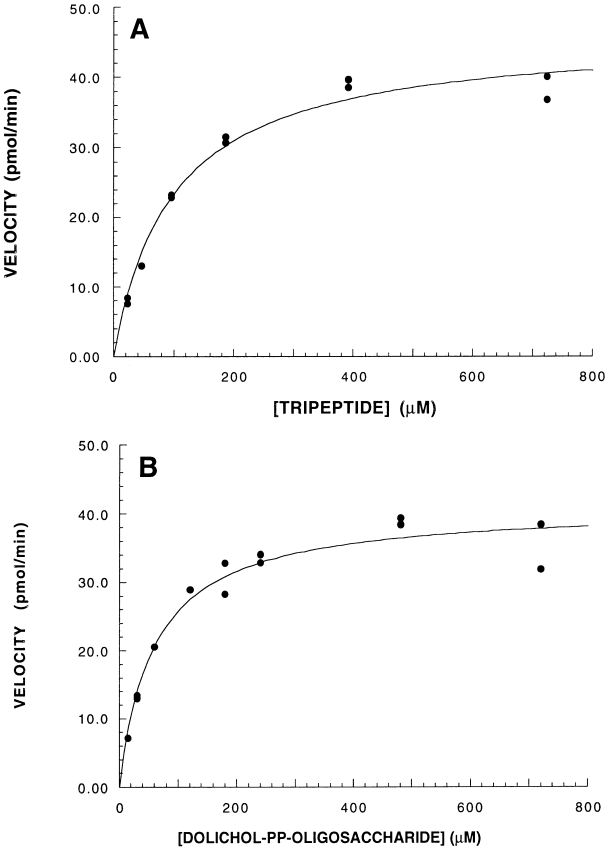

Figure 2. Velocity versus substrate concentration plots for solubilized OST. (A) Tripeptide as the variable substrate and pancreatic Dol-PP-OS kept constant at 118 mM. (B) Dol-PP-OS as the variable substrate and tripeptide kept constant at 682 mM.

Table 1. Kinetic constants for solubilized OST

Variable substrate[a]	Constant substrate[a]	[Constant substrate] (μM)	K_m (μM)	V_{max} (pmol/min)	V_{max}/K_m (rel)
Bz-NLT-NH_2	Dol-PP-DS	330	341.5 ± 2.0	16.2 ± 0.2	1.0
Bz-NLT-NH_2	Dol-PP-OS	118	127.6 ± 3.8	13.3 ± 0.1	2.2
Bz-NLT-NH_2	Dol-PP-OS[b]	50	45.1 ± 2.0	8.8 ± 0.1	4.2
Dol-PP-DS	Bz-NLT-NH_2	1250	64.8 ± 7.1	12.0 ± 0.2	3.9
Dol-PP-OS	Bz-NLT-NH_2	682	33.3 ± 1.6	12.5 ± 0.1	8.0

[a]Dol-PP-OS was isolated from bovine pancreas unless otherwise noted (see Experimental for details).
[b]Isolated from bovine thyroid.

possible that a minor component, Dol-PP-DS, of the heterogeneous Dol-PP-OS isolated from bovine tissue could be the active substrate in this mixture. However, based on comparison of data using synthetic Dol-PP-DS versus Dol-PP-OS (Table 1), this possibility seems unlikely. In other kinetic studies, K_m values for Dol-PP-OS and Dol-PP-DS were reported for solubilized OST.[12,39] While the authors also found a twofold difference between the K_m values, the numbers they report are significantly lower (ca. 1 μM) than those determined in this study. There are several possibilities for the discrepancy. Kinetic constants are usually determined at concentrations of the varied substrate, $[S] = 0.1–10\ K_m$. In the case of biosynthetic Dol-PP-OS and Dol-PP-DS used in the earlier research, low concentrations (≤1 μM) of the donor substrate may have led to errors in the determination of K_m for that substrate. Determining the concentration of the biosynthetic OST donor substrates is often not done or is done indirectly, possibly leading to erroneous results.[40] In addition, the difference may be due to the peptide substrate. The present study used a small tripeptide substrate whereas larger hexa- and heptapeptides were used in the previous work.[12,39] As discussed below, we have addressed the dependence of the peptide K_m on the lipidoligosaccharide substrate. However, it is not known if the length and amino acid composition of the peptide influences the K_m of the lipidoligosaccharide.

The values of K_m determined in this study for peptide acceptor substrates (Table 1) are consistent with most of those reported in the literature for similar peptides using Dol-PP-DS as the donor substrate. The tripeptides Bz-Asn-Leu-Thr-NHMe and Bz-Asn-Gly-Thr-NHMe have K_ms of 240 μM[18] and 220 μM,[21] in reasonable agreement with the value of 341 μM reported here. However, the peptide K_m is dependent on the size of the lipidoligosaccharide present. As the average length of the oligosaccharide moiety increases (Dol-PP-DS versus pancreas or thyroid Dol-PP-OS), the K_m for the tripeptide decreases up to eightfold. Previously reported values of K_m for acceptor peptides[12,41] using Dol-PP-OS as the donor substrate are difficult to compare with the data reported herein. The earlier work used biosynthetic Dol-PP-OS, the concentration of which is difficult to determine with certainty.[40] It has been suggested[12] that V_{max} for the peptide is also dependent on the lipidoligosaccharide in contrast to the data presented in Table 1. However, as noted above, the lipidoligosaccharides were calculated[40] to be present at concentrations (≤1 μM) well below the K_m values reported herein and saturating concentrations may not have been achieved.

The isolation and purification of Dol-PP-OS from bovine tissue in quantities sufficient for its use in a kinetic analysis of OST-catalyzed glycosylation is reported in this paper. Several postulated mechanisms reported to date for the OST-catalyzed glycosylation reaction are based on kinetics experiments using Dol-PP-DS as the oligosaccharide donor in order to obtain acceptor peptide K_m data.[18,21,42] Non-kinetics experiments using NMR techniques have called into question mechanisms requiring a specific secondary structure motif (e.g. Asx-turn[18]) for efficient peptide glycosylation.[43] The longer oligosaccharides evaluated in the present research promote a stronger interaction of the peptide substrate with the enzyme (K_m), but apparently are not necessary for placing or holding the peptide in the correct conformation for efficient transfer of the oligosaccharide to the peptide (V_{max}). Further application of this approach should allow for more detailed steady-state kinetics studies of OST-catalyzed glycosylation utilizing the lipidoligosaccharide, Dol-PP-OS, employed in vivo during the cotranslational glycosylation reaction that occurs in *N*-linked glycoprotein biosynthesis.

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40. In general, the 'specific activity' of biosynthetic [^{14}C]Dol-PP-OS has been determined by mild acid hydrolysis of the ^{14}C-labeled material to ^{14}C-labeled oligosaccharide containing a free reducing end that is then subjected to reduction by [^{3}H]$NaBH_4$, thus giving a doubly labeled oligosaccharide. Following extensive purification, the 'specific activity' of the original [^{14}C]Dol-PP-OS is obtained based on the specific activity of the purified [^{3}H, ^{14}C] oligosaccharide.[12]
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