^{13}C- and ^{15}N-Labeled Peptide Substrates as Mechanistic Probes of Oligosaccharyltransferase†

Tong Xu and James K. Coward*

Department of Chemistry, Interdepartmental Program in Medicinal Chemistry, and College of Pharmacy, The University of Michigan, Ann Arbor, Michigan 48109

Received August 7, 1997; Revised Manuscript Received October 1, 1997⊗

ABSTRACT: The carboxamide moiety that links the carbohydrate and protein moieties in N-linked glycoproteins has been unambiguously determined to arise intact from asparagine by the use of chemically synthesized Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-NH_2 as an oligosaccharyltransferase (OST) substrate. Bz-[4-^{13}C]Asn-Leu-Thr-NH_2 was also synthesized and used to evaluate a proposed mechanism of OST catalysis similar to that of glutamine-dependent amidotransferases using $^{15}NH_4OAc$ as a potential external nucleophile. Analysis of NMR and MS spectra of the isotopically labeled peptides and the resulting biosynthesized glycopeptides indicates that free $^{15}NH_3$ is not lost from the doubly labeled substrate during catalysis nor can exogenous $^{15}NH_3$ intercept any of several postulated enzyme-bound species. These results indicate that OST-catalyzed glycosylation does not follow a mechanism involving the transient generation of exchangeable "NH_3". Thus, in contrast to several glutamine-dependent amidotransferases, OST catalysis does not lead to transient scission of the asparagine β-carboxamide C−N bond. Together with previously published results, these data argue against nucleophilic activation of the asparagine β-carboxamide moiety being the underlying chemical mechanism for OST-catalyzed glycosylation of peptides.

Oligosaccharyltransferase (OST,[1] EC 2.4.1.119) is a membrane-bound, heterooligomeric enzyme that catalyzes the formation of N-linked glycoproteins (*1*, *2*). The glycosylation reaction is a cotranslational process which occurs shortly after the nascent peptide crosses the endoplasmic reticulum (ER) membrane. Although research on the biochemical properties of OST was initiated more than two decades ago, purification of OST has been accomplished only recently (*2*). The OST complex isolated from the yeast *Saccharomyces cerevisiae*, the subject of the research reported in this paper, consists of six polypeptides (*3*, *4*). During OST-catalyzed N-linked glycoprotein formation, the oligosaccharyl moiety from dolichyl-P-P-$(GlcNAc)_2Man_9Glc_3$ (**1a**, Dol-P-P-OS) is transferred to the β-amido group of an asparagine moiety in the growing polypeptide chain (Figure 1). The polypeptide chain must contain an Asn-X-Ser/Thr sequon, where X can be any natural amino acid except proline, for glycosylation to occur (*5*, *6*). Suitably protected tripeptides containing the required sequon have been shown to be good OST substrates *in vitro* (*7*−*10*). Our previous research also showed that a chemically synthesized chitobiose-linked lipid pyrophosphate, dolichyl-P-P-$(GlcNAc)_2$ (**1b**, Dol-P-P-DS), can substitute for Dol-P-P-OS as the OST substrate (*11*, *12*). Therefore, both substrates of OST are accessible in reasonable amounts through chemical synthesis.

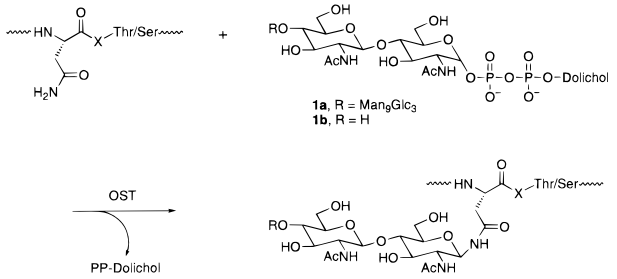


FIGURE 1: OST-catalyzed formation of N-linked glycoproteins.

Although OST was first successfully purified in 1992, limited mechanistic studies of the OST-catalyzed reaction have been pursued since ca. 1980 using crude, solubilized enzyme. In the OST-catalyzed reaction, the side chain carboxamide of asparagine apparently displaces dolichol pyrophosphate through a direct nucleophilic attack to form a new glycosyl (N−C) bond of the growing N-linked glycoprotein (Figure 1). However, the nucleophilicity of the carboxamide is insufficient to allow such an S_N2 attack under nonenzymatic conditions. Thus, any proposed mechanism must explain activation of the side chain carboxamide of asparagine during OST catalysis. Bause and Legler (*13*) first proposed an intramolecular deprotonation mechanism involving an active site base. More recently, Imperiali and coworkers (*1*, *14*) proposed the "Asx-turn" conformation as the basis for carboxamide activation.

Since OST catalyzes a reaction similar to many glutamine-dependent amidotransferases (*15*, *16*), we have investigated several variants of a mechanism involving nucleophilic activation of the asparagine carboxamide leading to the

† This work was supported in part by funds from the Vahlteich Research Fund, College of Pharmacy, The University of Michigan. Mass spectral data were obtained at the Michigan State University Mass Spectrometry Facility which is supported, in part, by a grant (DRR-00480) from the Biotechnology Research Technology Program, National Center for Research Resources, National Institutes of Health.

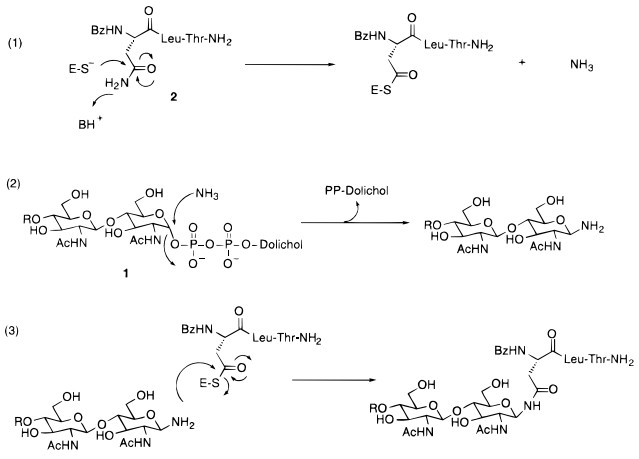
* To whom correspondence should be addressed. Telephone: (313) 936-2843. Fax: (313) 647-4865.

⊗ Abstract published in *Advance ACS Abstracts*, November 1, 1997.

[1] Abbreviations: Boc, *tert*-butoxycarbonyl; Bz, benzoyl; Glc, glucose; GlcNAc, *N*-acetyl-D-glucosamine; Dol-P-P-DS, lipid disaccharide; Dol-P-P-OS, lipid oligosaccharide; Man, mannose; MMTS, methyl methanethiolsulfonate; OST, oligosaccharyltransferase; pCMB, *p*-(chloromercuri)benzoic acid.

S0006-2960(97)01951-X CCC: $14.00

Scheme 1: Proposed OST Catalytic Mechanism by Analogy with Glutamine-Dependent Amidotransferases



generation of activated aspartate and nascent NH_3, or the equivalent tetrahedral intermediate, at the enzyme active site (*10*, *17*). By extrapolation from the glutamine-dependent amidotransferases, it is reasonable to hypothesize that the carboxamide of asparagine might be activated by an active site thiol group to form an aspartate β-thiol ester and nascent NH_3 at the OST active site. The nascent NH_3 could then displace dolichol pyrophosphate from the anomeric carbon of *N*-acetylglucosamine to form a chitobiosylamine which then could attack the activated aspartate β-thiol ester to form the glycoprotein linkage bond (Scheme 1). Involvement of the critical cysteine residue is suggested by the fact that thiol reagents such as MMTS (*18*) (T. Xu and J. K. Coward, unpublished data) and pCMB (Y.-L. Liu, L. Schretzman, and J. K. Coward, unpublished data) inhibit OST glycosylation activity. Two other thiol reagents, iodoacetate and iodoacetamide, have no inhibitory effect (Y.-L. Liu, L. Schretzman, and J. K. Coward, unpublished data). However, experiments of this type involving nonspecific, nonactive site-directed alkylating agents do not address the role(s), if any, of the targeted amino acids in catalysis.

To study the mechanism proposed in Scheme 1, it is first necessary to establish unambiguously the origin of the carboxamide linkage bond in the glycosylated product. Since adjacent nuclei with a spin of $^1/_2$ (e.g., ^{1}H, ^{13}C, and ^{15}N) are coupled in NMR spectroscopy, a peptide substrate containing [4-^{13}C, ^{15}N]asparagine should show ^{13}C$-^{15}$N coupling in both ^{13}C and ^{15}N NMR spectra. Following OST-catalyzed glycosylation reaction using synthetic Dol-P-P-DS and Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-NH_2 (**2a**) (eq 1), the glycopeptide product can be isolated for spectral studies by methods previously established in our laboratory (*10*, *11*, *17*). Retention of the ^{13}C$-^{15}$N coupling in the ^{13}C NMR spectra of the biosynthesized glycopeptide product **3a** would prove that the side chain carboxamide of asparagine is preserved in the glycosylated product. Conversely, washout of ^{15}N during catalysis would provide evidence that any free "NH_3" formed (Scheme 1) can be freely exchanged with bulk solvent and reagents, including unlabeled NH_3 present in the assay mixture. The use of ^{13}C$-^{15}$N coupling of adjacent nuclei for mechanistic study has been used previously in the area of secondary metabolite biosynthesis (*19*, *20*).

Glycoprotein biosynthesis in intact cells is associated with the release of free oligosaccharides via a "Dol-P-P-OS hydrolase" activity. This activity is thought to be associated with the OST protein complex (*21*) and may play a regulatory role in the cell (*22*). This apparently wasteful hydrolysis of Dol-P-P-OS can be viewed as the adventitious interception of an enzyme-bound species (Dol-P-P-OS) by an external nucleophile (H_2O). Thus, it is reasonable to postulate that other external nucleophiles such as NH_3 may have access to enzyme-bound Dol-P-P-OS during OST-catalyzed glycosylation (Scheme 1). A labeled peptide substrate, Bz-[4-^{13}C]-Asn-Leu-Thr-NH_2 (**2b**), has been used as a probe of this aspect of the mechanism. If the OST active site is accessible to external nucleophiles, and the mechanism shown in Scheme 1 is operative, the presence of ^{15}N-labeled NH_3 (as $^{15}NH_4OAc$) in the enzyme assay (eq 2) would result in the formation of the same ^{13}C- and ^{15}N-labeled glycopeptide described above. Purification and spectroscopic analysis of the biosynthesized glycopeptide **3b** should allow us to evaluate this aspect of the proposed mechanism. Addition of exogenous nucleophiles to trap reactive intermediates has extensive precedent in many areas of mechanistic enzymology (*23*, *24*).

$$\underset{\textbf{2a}}{\text{Bz-[4-}^{13}\text{C, }^{15}\text{N]Asn-Leu-Thr-NH}_2} + \underset{\textbf{1b}}{\text{Dol-P-P-DS}} \xrightarrow{\text{OST}} \underset{\textbf{3a}}{\text{glycopeptide}} \quad (1)$$

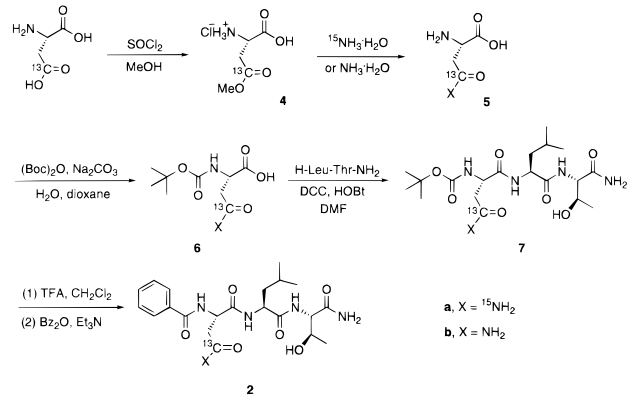
$$\underset{\textbf{2b}}{\text{Bz-[4-}^{13}\text{C]Asn-Leu-Thr-NH}_2} + \underset{\textbf{1b}}{\text{Dol-P-P-DS}} \xrightarrow[^{15}\text{NHOAc}]{\text{OST}} \underset{\textbf{3b}}{\text{glycopeptide}} \quad (2)$$

MATERIALS AND METHODS

General. ^{1}H and ^{13}C NMR spectral data are reported in parts per million downfield from TMS. ^{15}N NMR chemical shift data are reported relative to external aqueous K^{13}C^{15}N, which was assigned a chemical shift of 101 ppm relative to $MeNO_2$ (*25*). L-[4-^{13}C]Aspartic acid ($\geq$99 at. % ^{13}C), [^{15}N]ammonium hydroxide (99.1 at. % ^{15}N), and [^{15}N]NH_4-OAc (99.6 at. % ^{15}N) were purchased from Isotec, Inc. Unless otherwise indicated, reagents and solvents were obtained from commercial sources and were used without further purification. Reversed-phase HPLC analyses were performed on a Waters liquid chromatography system (6100A and 510 pumps), Waters μBondpak C_{18}, 300 Å, 3.9 $\times$ 150 mm column or Rainin Microsorb-MV 5 μm C_{18}, 300 Å, 4.6 $\times$ 250 mm column, and monitored using a Waters 996 diode array spectrometer equipped with Millennium software. P_{40} yeast microsomes and [^{3}H]Dol-P-P-DS were prepared as described by Clark et al. (*10*) and Lee and Coward (*11*), respectively. *N*-Bz-Asn-Leu-Thr-NH_2 was synthesized as described previously by Clark et al. (*10*). Stable isotopically labeled amino acids and peptides were synthesized on the basis of the previous method of Lee and Coward (*17*). Dol-P-P-DS was synthesized as previously described (*11*, *12*), and its concentration was determined by high-pH anion-exchange chromatography (HPAEC) (*26*) following hydrolysis to glucosamine.

Synthesis of N-Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-NH_2 and N-Bz-[4-^{13}C]Asn-Leu-Thr-NH_2. Although a search of Chemical

Scheme 2: Synthesis of Stable Isotope-Labeled OST Peptide Substrates



Abstracts for this doubly labeled asparagine led to a single reference (*27*), no synthesis of the target compound was described. We have previously described the synthesis of three deuterium-labeled asparagine-containing peptides for use as OST substrates (*17*). The synthesis of ^{13}C- and ^{15}N-labeled peptide and the ^{13}C-labeled peptide required in the present research was accomplished using these methods (Scheme 2). L-[4-^{13}C]Aspartic acid was first esterified regiospecifically at the β-carboxylic acid, and the resulting β-methyl ester reacted with either concentrated $^{15}NH_4OH$ or NH_4OH to give L-[4-^{13}C, ^{15}N]asparagine (**5a**) or L-[4-^{13}C]-asparagine (**5b**), respectively. The labeled asparagine was protected at the N terminus by reaction with $(Boc)_2O$, and the labeled *N*-Boc-L-asparagine (**6**) was then coupled with H-Leu-Thr-NH_2·TFA to give labeled *N*-Boc-Asn-Leu-Thr-NH_2 (**7**). Deprotection followed by N-benzoylation of the N terminus gave the desired labeled peptide substrates **2a** and **2b**. Synthetic details and spectral data can be found in the Supporting Information.

Standard OST Assay Using [^{3}H]Dol-P-P-DS. The synthetic peptides **2a** and **2b** were tested as OST substrates using the previously described method (*11*). The assay mixture contained ca. 6000 dpm [^{3}H]Dol-P-P-DS, 50 mM Tris (pH 7.5), 1% Triton X-100, 1 mM $MnCl_2$, 360 μM *N*-Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-NH_2 or *N*-Bz-[4-^{13}C]Asn-Leu-Thr-NH_2 in DMSO (final [DMSO] = 5% v/v), and 875 μg of P_{40} yeast microsome (*10*) in a total volume of 100 μL. The assay mixture was shaken at 250 rpm for 2 h at room temperature, and then the reaction was quenched by the addition of 3 mL of cold $CHCl_3$/MeOH (v/v = 3/2). The mixture was allowed to set on ice for 30 min, and the layers were separated by centrifugation at 1000*g* for 15 min. The supernatant was removed and extracted with 1 mL of 4 mM $MgCl_2$. The biphasic mixture was thoroughly agitated (Vortex) and then centrifuged at 1000*g* for another 15 min. The upper aqueous layer containing the water soluble ^{3}H-labeled glycopeptide was carefully removed. A significant increase in ^{3}H radioactivity observed in the aqueous layer compared with that observed in the aqueous layer from a control assay not containing the peptide substrate provided evidence of the formation of ^{3}H-labeled glycopeptide (*11*).

*Biosynthesis of Glycopeptide Using N-Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-NH_2 (**2a**) and Chemically Synthesized Dol-P-P-DS*. Each preparative OST reaction mixture contained 66 μM chemically synthesized Dol-P-P-DS, 50 mM Tris (pH 7.5), 1% Triton X-100, 1 mM $MnCl_2$, 360 μM *N*-Bz-[4-^{13}C, ^{15}N]-Asn-Leu-Thr-NH_2 (**2a**) in DMSO (final [DMSO] = 5% v/v), and 875 μg of P_{40} yeast microsome in a total volume of 100 μL. Multiple reactions (160) were carried out as described above, and the aqueous layers were combined and concentrated in vacuo.

*Biosynthesis of Glycopeptide Using N-Bz-[4-^{13}C]Asn-Leu-Thr-NH_2 (**2b**) and Chemically Synthesized Dol-P-P-DS with $^{15}NH_4OAc$ as the Potential External Nucleophile*. Each preparative OST reaction mixture contained 66 μM chemically synthesized Dol-P-P-DS, 50 mM Tris (pH 7.5), 1% Triton X-100, 1 mM $MnCl_2$, 360 μM *N*-Bz-[4-^{13}C]Asn-Leu-Thr-NH_2 (**2b**) in DMSO (final [DMSO] = 5% v/v), 360 mM $^{15}NH_4OAc$, and 875 μg of P_{40} yeast microsome in a total volume of 100 μL. Multiple reactions (260) were carried out as described above, and the aqueous layers were combined and concentrated in vacuo.

Purification and NMR Study of the Biosynthesized Glycopeptides. The concentrated solution containing each biosynthesized glycopeptide was purified by HPLC involving multiple runs using a Waters liquid chromatography system with Millennium software and a Rainin Microsorb column. A gradient profile of 10 to 25% MeOH over 20 min followed by a 25% MeOH isocratic condition was employed. The unreacted peptide substrate (**2a** and **2b**, t_R = 69.0 min) and the biosynthesized glycopeptide (**3a** and **3b**, t_R = 76.5 min) were readily separated by this procedure. Fractions containing the glycopeptide were combined and concentrated in vacuo. The dry residue of each biosynthetic glycopeptide was dissolved in 400 μL of D_2O/CD_3OD (v/v, 1/1) for NMR studies.

Analysis of the Mass Spectroscopy Data of the Labeled Synthetic Peptides and Biosynthesized Glycopeptides. FAB^+ MS data of the labeled peptides **2a** and **2b** and the biosynthesized glycopeptides **3a** and **3b** were obtained. The theoretical ion distribution was calculated using a JEOL DA 5000 data system (JEOL, USA, Peabody, MA). Calculation of the stable isotope enrichments of **2a**, **2b**, **3a**, and **3b** was based on the theoretical ion distribution and the observed relative intensities of the molecular ions. For example, the unlabeled Bz-Asn-Leu-Thr-NH_2 (MH^+ $C_{21}H_{32}N_5O_6$, 450) has the following theoretical ion distribution based on the known natural abundance of all isotopes: *m*/*z* 450, 451, 452; ratio, 100, 26.06, 4.49. The relative intensities of the related peaks observed for **2a** (Figure 4A) are *m*/*z* 450, 451, 452; relative intensity, 4.20, 3.25, 47.14. While the *m*/*z* 450 peak results only from the molecular ion of unlabeled Bz-Asn-Leu-Thr-NH_2, the *m*/*z* 451 peak is a combination of the A + 1 peak of unlabeled Bz-Asn-Leu-Thr-NH_2 and the molecular ion peak of Bz-[4-^{13}C]Asn-Leu-Thr-NH_2.[2] Similarly, the *m*/*z* 452 peak is composed of the A + 2 peak of unlabeled Bz-Asn-Leu-Thr-NH_2, the A + 1 peak of Bz-[4-^{13}C]Asn-Leu-Thr-NH_2, and the molecular ion of Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-NH_2. If the relative content of the unlabeled Bz-Asn-Leu-Thr-NH_2 (MH^+, 450) is defined as *X*, that of Bz-[4-^{13}C]Asn-Leu-Thr-NH_2 (MH^+, 451) as *Y*, and that of Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-NH_2 (MH^+, 452) as *Z*, it follows that for **2a** $X = 4.20$, $Y + 0.2606X = 3.25$, and $Z + 0.2606Y + 0.0449X = 47.14$. The stable isotope distribution in **2a** can then be calculated: unlabeled Bz-Asn-Leu-Thr-NH_2 = $X/(X + Y + Z)$ = 7.96%, singly labeled Bz-[4-^{13}C]Asn-Leu-Thr-

[2] It should be noted that Bz-[4-^{15}N]Asn-Leu-Thr-NH_2, which would also give rise to a *m*/*z* 451 peak, is a very minor component and can be ignored in these calculations.

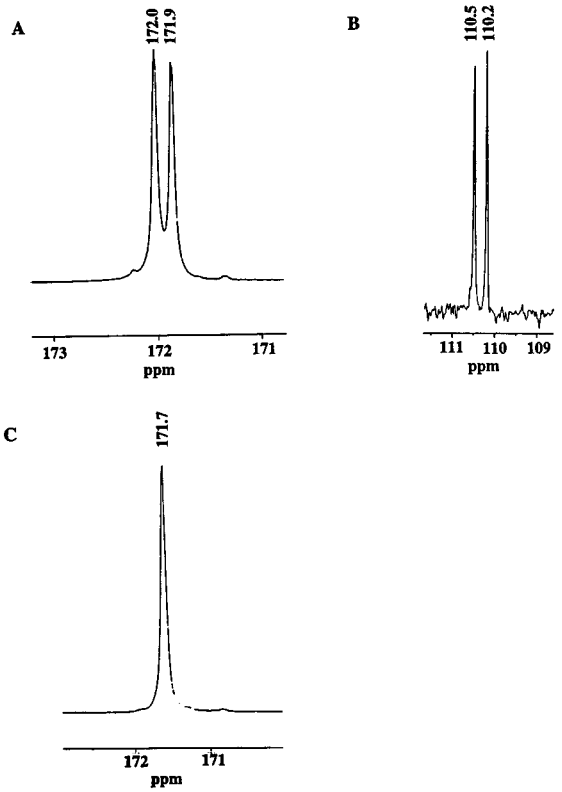


FIGURE 2: (A) ^{13}C and (B) ^{15}N NMR spectra of the synthetic peptide **2a** and (C) ^{13}C NMR spectrum of synthetic peptide **2b**.

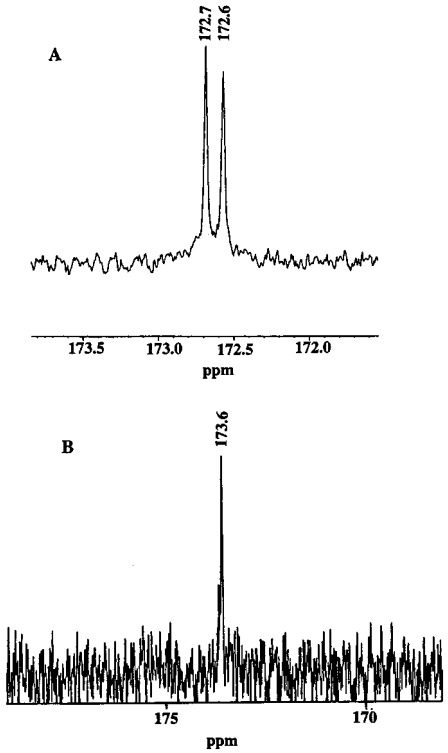


FIGURE 3: ^{13}C NMR spectra of the biosynthesized glycopeptides (A) **3a** and (B) **3b**.

$NH_2 = Y/(X + Y + Z) = 4.09\%$, and doubly labeled Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-$NH_2 = Z/(X + Y + Z) = 87.9\%$.

In a similar fashion, the unlabeled glycopeptide Bz-Asn-$(GlcNAc)_2$-Leu-Thr-NH_2 (MH^+ $C_{37}H_{58}N_7O_{16}$, 856) has the following theoretical ion distribution: *m/z* 856, 857, 858; relative intensity, 100, 45.48, 13.38. On the basis of the MS data, the stable isotope distribution of the glycopeptide **3a** was calculated to be 10.2% for unlabeled Bz-Asn-$(GlcNAc)_2$-Leu-Thr-NH_2, 8.06% for singly labeled Bz-[4-^{13}C]Asn$(GlcNAc)_2$-Leu-Thr-NH_2, and 81.7% for doubly labeled Bz-[4-^{13}C, ^{15}N]Asn$(GlcNAc)_2$-Leu-Thr-NH_2. The isotope distributions of **2b** and **3b** were calculated using the same method, and the results are shown, together with those of **2a** and **3a**, in Table 1.

RESULTS AND DISCUSSION

The syntheses of Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-NH_2 (**2a**) and Bz-[4-^{13}C]Asn-Leu-Thr-NH_2 (**2b**) were completed following the established method (*11*) shown in Scheme 2. A clear doublet resulting from labeled $^{13}C-^{15}N$ coupling was observed in the ^{13}C and ^{15}N NMR spectra of compounds **5**−**7a** and **2a**. The final doubly labeled peptide **2a** showed a $^{13}C-^{15}N$ coupling constant of 14.8 Hz in its ^{13}C NMR (Figure 2A) and 14.6 Hz in its ^{15}N NMR spectra (Figure 2B), as expected for adjacent ^{13}C and ^{15}N nuclei (*25*). Interestingly, the β-carbon of asparagine was observed as a doublet of doublets because of its coupling with the enriched ^{13}C and ^{15}N nuclei (spectra not shown). A large single peak was observed in the ^{13}C NMR spectra of **4**−**7b** and **2b** (Figure 2C) due to the single ^{13}C-enriched carbon at C-4. Compounds **2a** and **2b** were shown to be equally good OST substrates, comparable to unlabeled standard peptide Bz-Asn-Leu-Thr-NH_2, using [^{3}H]Dol-P-P-DS and microsomal enzyme (*11*).

To investigate the proposed mechanism shown in Scheme 1, sufficient biosynthetic glycopeptide must be obtained for ^{13}C NMR and mass spectroscopy studies. Initial NMR instrument sensitivity studies showed that approximately 200 nmol of glycopeptide would be needed to observe the $^{13}C-^{15}N$ coupled doublet in the ^{13}C NMR spectra (8 h scan) with acceptable signal:noise. Initially, OST assay studies were performed using chemically synthesized Dol-P-P-DS (**1b**) and [^{14}C]Bz-Asn-Leu-Thr-NH_2 to optimize the yield of the desired glycopeptides. These experiments led us to choose 66 μM **1b** and 360 μM **2** for use in multiple reactions to obtain enough biosynthetic glycopeptide for NMR analysis.[3] Under these conditions, OST-catalyzed glycosylation of **2a** led to 235 nmol (22.3% based on Dol-P-P-DS) of biosynthesized glycopeptide **3a**. The biosynthesized glycopeptide **3a** showed a clear doublet at δ 172.6 with a J_{CN} of 14.5 Hz in the ^{13}C NMR spectrum (Figure 3A), indicating retention of both atoms of the asparagine side chain carboxamide during OST-catalyzed N-linked glycoprotein synthesis. Unfortunately, the decreased sensitivity of the ^{15}N nucleus precluded acquisition of satisfactory ^{15}N NMR spectral data for glycopeptide **3a** even after prolonged (40 h) acquisition times.

In the case of PRPP amidotransferase, K_m of free ammonia is 400 times that of glutamine (*28*, *29*). Therefore, a high concentration of exogenous $^{15}NH_4OAc$ is desired in order to increase the chance of intercepting any intermediate(s)

[3] It is conceivable that a peptide substrate such as **2** could react with a nucleophilic residue at the OST active site (Scheme 1) in the absence of the oligosaccharide donor, **1**. Subsequent hydrolysis of the resulting acyl enzyme would lead to *N*-Bz-Asp-Leu-Thr-NH_2. However, HPLC analysis of an enzyme reaction carried out in the absence of **1b** failed to indicate any production of the Asp-containing tripeptide under these conditions.

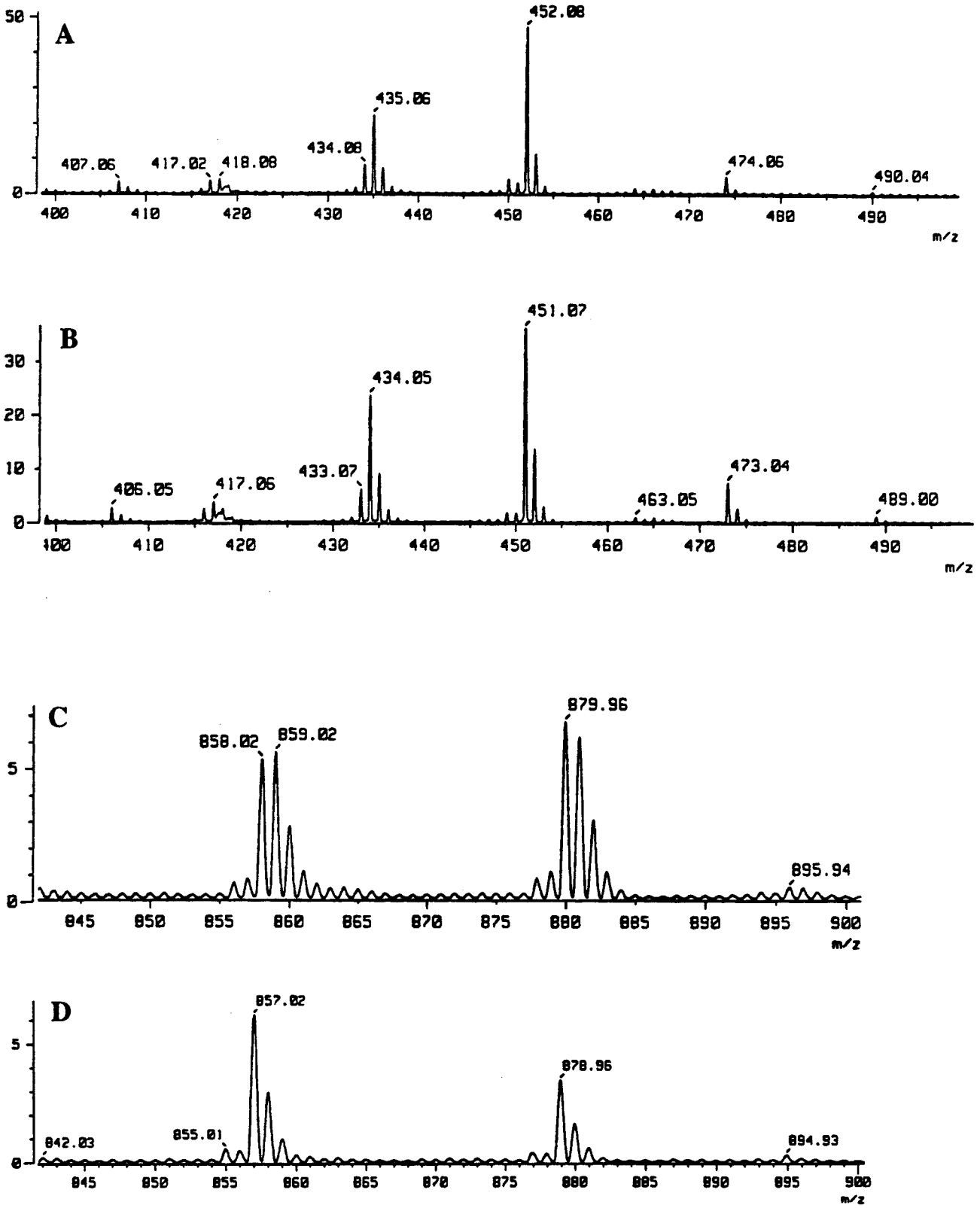


FIGURE 4: Portions of mass spectra of (A) synthetic peptide **2a**, (B) synthetic peptide **2b**, (C) biosynthetic glycopeptide **3a**, and (D) biosynthetic glycopeptide **3b**.

formed during OST catalysis. This, in turn, would lead to $^{13}C-^{15}N$ coupling if the OST catalytic mechanism parallels that of glutamine-dependent amidotransferases (Scheme 1). $^{15}NH_4OAc$ at a concentration of 360 mM, 1000 times that of the peptide substrate Bz-[4-^{13}C]Asn-Leu-Thr-NH_2 (**2b**), was used in the reaction. However, under this condition, the glycosylation yield decreased to 60% of that obtained in the absence of ammonium salt. Although reaction at higher pH would ensure an increased concentration of NH_3, the required nucleophilic base, preliminary studies showed that the yield of the glycopeptide decreased significantly at higher pH. Therefore, physiological pH 7.5 was used in the multiple reactions carried out to obtain sufficient glycopeptide for NMR analysis; 170.2 nmol of glycopeptide **3b** (9.9% based on Dol-P-P-DS) was obtained after two HPLC purifications. The ^{13}C NMR spectrum of **3b** showed a clear singlet at δ

Table 1: Quantitative Mass Spectral Analysis of Peptides **2a** and **2b** and the Resulting Biosynthetic Glycopeptides **3a** and **3b**

	peak relative intensity			isotope distribution (%)		
compound	M^+	$(M+1)^+$	$(M+2)^+$	$^{12}C/^{14}N$	$^{13}C/^{14}N$	$^{13}C/^{15}N$
2a	4.20	3.25	47.1	7.96	4.09	87.9
3a	0.62	0.77	5.25	10.2	8.06	81.7
2b	1.71	36.0	13.5	4.33	85.1	10.6
3b	0.51	6.25	2.98	7.61	89.8	2.57

173.6 (Figure 3B), indicating that exogenous $^{15}NH_3$ cannot be involved in the synthesis of Bz-[4-^{13}C, ^{15}N]Asn(GlcNAc)$_2$-Leu-Thr-NH_2 from **2b** during OST catalysis.

While these NMR studies of the two biosynthesized glycopeptides **3a** and **3b** provide strong evidence that there is little mechanistic similarity between OST and glutamine-dependent amidotransferases (Scheme 1), quantitative analysis of the mass spectral data obtained for **2a**, **2b**, **3a**, and **3b** further strengthened this conclusion (Figure 4). In Table 1, **2a** is shown to be 87.9% enriched in the doubly labeled isotopomer, Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-NH_2, while the resulting glycopeptide **3a** has a ^{13}C, ^{15}N enrichment of 81.7%. However, this slight decrease is not accompanied by an increase of Bz-[4-^{13}C]Asn(GlcNAc)$_2$-Leu-Thr-NH_2 (MH^+, 857) content in **3a** as would be expected if free exogenous $^{14}NH_3$ were exchanging with the ^{15}N originating in **2a**. The enrichment of Bz-[4-^{13}C]Asn(GlcNAc)$_2$-Leu-Thr-NH_2 (8.06%) in **3a** is similar to that of Bz-[4-^{13}C]Asn-Leu-Thr-NH_2 (4.09%) in **2a**.

A similar analysis can be applied to glycopeptide **3b**, obtained from the OST-catalyzed glycosylation of **2b** in the presence of excess $^{15}NH_4OAc$. As shown in Table 1, **2b** is 85.1% enriched in the singly labeled isotopomer, Bz-[4-^{13}C]-Asn-Leu-Thr-NH_2. A decrease in the Bz-[4-^{13}C]Asn-(GlcNAc)$_2$-Leu-Thr-NH_2 content coupled with an increase of Bz-[4-^{13}C, ^{15}N]Asn(GlcNAc)$_2$-Leu-Thr-NH_2 observed in **3b** is expected if external $^{15}NH_3$ can access the OST active site and exchange with any nascent ammonia produced during catalysis. However, the resulting glycopeptide **3b** shows a Bz-[4-^{13}C]Asn(GlcNAc)$_2$-Leu-Thr-NH_2 enrichment of 89.8%, very similar to that of **2b**. Furthermore, the content of Bz-[4-^{13}C, ^{15}N]Asn(GlcNAc)$_2$-Leu-Thr-NH_2 (2.6%) in **3b** is much smaller than the content of Bz-[4-^{13}C, ^{15}N]-Asn-Leu-Thr-NH_2 (10.6%) in **2b**, further indicating that there is no Bz-[4-^{13}C, ^{15}N]Asn(GlcNAc)$_2$-Leu-Thr-NH_2 formed during the OST-catalyzed glycosylation of **2b** even in the presence of an external source of $^{15}NH_3$.

In conclusion, ^{13}C-/^{15}N- and ^{13}C-labeled tripeptide substrates were synthesized and used with chemically synthesized Dol-P-P-DS for mechanistic studies of the OST-catalyzed reaction. Retention of the side chain carboxamide of asparagine in the glycopeptide product was unambiguously demonstrated by the observation of $^{13}C-^{15}N$ coupling in the ^{13}C NMR spectra of the biosynthetic glycopeptide when Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-NH_2 (**2a**) was used as the peptide substrate. The inability of ^{15}N from a labeled exogenous nucleophile ($^{15}NH_4OAc$) to intercept any intermediate derived from **2b** and incorporate it into the glycopeptide product suggests that OST catalysis does not follow the mechanism established for several glutamine-dependent amidotransferases (*15*). Stoker et al. (*30*) have recently used ^{13}C- and ^{15}N-labeled glutamine to study the catalytic mechanism of *Escherichia coli* asparagine synthetase B, a glutamine-dependent amidotransferase. Through heavy atom kinetic isotope effect results, they proposed that free ammonia does not form in the enzyme active site during nitrogen transfer. Similarly, our two labeled peptides, Bz-[4-^{13}C]Asn-Leu-Thr-NH_2 and Bz-[4-^{13}C, ^{15}N]Asn-Leu-Thr-NH_2, may also act as useful kinetic probes for further mechanistic studies of the OST-catalyzed reactions.

ACKNOWLEDGMENT

We thank Mr. Xinggao Fang for the synthesis of Dol-P-P-DS, Dr. Barbara Gibbs for the synthesis of [^{14}C]Bz-Asn-Leu-Thr-NH_2, Dr. Kevin Rice and Mr. Hayden Thomas for the determination of the Dol-P-P-DS concentration using their DIONEX system, Dr. Scott Woehler for his help with the ^{13}C and ^{15}N NMR experiments, and Ms. Carol Capelle for careful preparation of the manuscript. We also thank Dr. Barbara Gibbs for her helpful discussions during the course of this research.

SUPPORTING INFORMATION AVAILABLE

Details of the syntheses of **2a**, **2b**, and their precursors (6 pages). Ordering information is given on any current masthead page.

REFERENCES

1. Imperiali, B., and Hendrickson, T. L. (1995) *Bioorg. Med. Chem. 3*, 1565−1578.
2. Silberstein, S., and Gilmore, R. (1996) *FASEB J. 10*, 849−858.
3. Kelleher, D. J., and Gilmore, R. (1994) *J. Biol. Chem. 269*, 12908−12917.
4. Pathak, R., and Imperiali, B. (1997) *Arch. Biochem. Biophys. 338*, 1−6.
5. Gavel, Y., and von Heijne, G. (1990) *Protein Eng. 3*, 433−442.
6. Marshall, R. D. (1972) *Annu. Rev. Biochem. 41*, 673−702.
7. Hart, G. W., Brew, K., Grant, G. A., Bradshaw, R. A., and Lennarz, W. J. (1979) *J. Biol. Chem. 254*, 9747−9753.
8. Welply, J. K., Shenbagamurthi, P., Lennarz, W. J., and Naider, F. (1983) *J. Biol. Chem. 258*, 11856−11863.
9. Rathod, P. K., Tashjian, A. H., Jr., and Abeles, R. H. (1986) *J. Biol. Chem. 261*, 6461−6469.
10. Clark, R. S., Banerjee, S., and Coward, J. K. (1990) *J. Org. Chem. 55*, 6275−6285.
11. Lee, J., and Coward, J. K. (1992) *J. Org. Chem. 57*, 4126−4135.
12. Fang, X., Gibbs, B. S., and Coward, J. K. (1995) *Bioorg. Med. Chem. Lett. 5*, 2701−2706.
13. Bause, E., and Legler, G. (1981) *Biochem. J. 195*, 639−644.
14. Imperiali, B., Shannon, K. L., Unno, M., and Rickert, K. W. (1992) *J. Am. Chem. Soc. 114*, 7944−7945.
15. Zalkin, H. (1993) *Adv. Enzymol. Relat. Areas Mol. Biol. 66*, 203−309.
16. Schendel, F. J., Cheng, Y. S., Otvos, J. D., Wehrli, S., and Stubbe, J. (1988) *Biochemistry 27*, 2614−2623.
17. Lee, J., and Coward, J. K. (1993) *Biochemistry 32*, 6794−6801.
18. Pathak, R., Hendrickson, T. L., and Imperiali, B. (1995) *Biochemistry 34*, 4179−4185.
19. Gould, S. J., Chang, C. C., Darling, D. S., Roberts, J. D., and Squillacote, M. (1980) *J. Am. Chem. Soc. 102*, 1707−1712.
20. Parry, R. J., Li, Y., and Lii, F.-L. (1992) *J. Am. Chem. Soc. 114*, 10062−10065.
21. Anumula, K. R., and Spiro, R. G. (1983) *J. Biol. Chem. 258*, 15274−15282.
22. Spiro, M. J., and Spiro, R. G. (1991) *J. Biol. Chem. 266*, 5311−5317.

23. Braun, C., Lindhorst, T., Madsen, N. D., and Withers, S. G. (1996) *Biochemistry 35*, 5458−5463.
24. Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, W. H. Freeman & Co., San Francisco.
25. Witanowski, M., and Webb, G. A. (1973) *Nitrogen NMR*, Plenum Publishing Co., London and New York.
26. Lee, Y. C. (1990) *Anal. Biochem. 189*, 151.
27. Schaefer, J., Stejskal, E. O., Garbow, J. R., and McKay, R. A. (1984) *J. Magn. Reson. 59*, 150−156.
28. Wyngaarden, J. B., and Ashton, D. M. (1959) *J. Biol. Chem. 234*, 1492−1496.
29. Hartman, S. C. (1963) *J. Biol. Chem. 238*, 3024−3035.
30. Stoker, P. W., O'Leary, M. H., Boehlein, S. K., Schuster, S. M., and Richards, N. G. J. (1996) *Biochemistry 35*, 3024−3030.
31. Schwarz, H., Bumpus, F. M., and Page, I. H. (1957) *J. Am. Chem. Soc. 79*, 5697−5703.
32. Gani, D., Young, D. W., Carr, D. M., Poyser, J. P., and Sadler, I. H. (1983) *J. Chem. Soc., Perkin Trans 1*, 2811−2814.
33. Beyerman, H. C., Boers-Boonekamp, C. A. M., and Maassen van den Brink-Zimmermannová, H. (1968) *Rec. Trav. Chim. 87*, 257−273.

BI9719511