Metal Ion Dependence of Oligosaccharyl Transferase: Implications for Catalysis[†]

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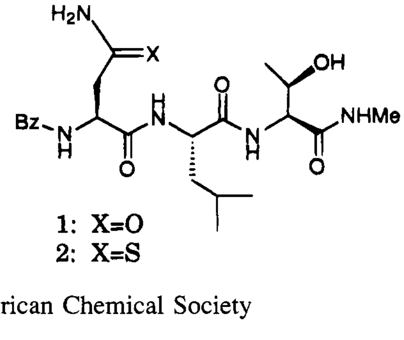
ABSTRACT: Oligosaccharyl transferase activity exhibits an absolute requirement for certain divalent metal cations. Studies with reconstituted enzyme suggest a preference for metal ions that can adopt an octahedral coordination geometry. In order to gain insight into the specific role of the metal cation in catalysis, we have investigated the influence of the metal cofactor on catalytic turnover of the tripeptide substrate Bz-Asn-Leu-Thr-NHMe (**1**) and a closely related sulfur-containing analog, Bz-Asn(γS)-Leu-Thr-NHMe (**2**). The metal ion substitution studies reveal that **1** is effectively turned over in the presence of several metal ions (Mn^{2+}, Fe^{2+}, Mg^{2+}, and Ca^{2+}). In contrast, **2** is only glycosylated in the presence of the thiophilic metal cations manganese and iron. When the enzyme is reconstituted with the oxophilic cations magnesium and calcium, **2** shows minimal substrate behavior. With the amide substrate **1**, the distinct preference for manganese over magnesium may argue against direct coordination of the metal to the lipid-linked substrate pyrophosphate moiety. This fact, together with the comparative studies with asparagine- and thioasparagine-containing tripeptides, implicates the metal cofactor in a role that places it proximal to the peptide binding site.

Asparagine-linked glycosylation is essential for the structural and functional integrity of many eukaryotic proteins. This glycosylation is mediated by the membrane-bound enzyme oligosaccharyl transferase (OT)[1] in the lumen of the endoplasmic reticulum. The reaction involves transfer of a complex, branched oligosaccharide $(GlcNAc)_2(Man)_9(Glc)_3$ from a dolichol pyrophosphate donor to the carboxamide nitrogen of asparagine within the consensus sequence -Asn-Xaa-Ser/Thr- (Presper & Heath, 1985). Since this cotranslational event represents the first committed step in the biosynthesis of all *N*-linked protein glycoconjugates, an understanding of the mechanism of the reaction is critical.

A complete consideration of the N-linked glycosylation process rests on the elucidation of the role of the necessary cofactors in the process. OT manifests an absolute requirement for certain divalent metal cations for activity. In the native enzyme, Mn^{2+} is considered to be the endogenous metal ion (Kaufman et al., 1994); however, in reconstitution experiments, magnesium can be substituted for manganese with the regeneration of approximately one-third of the native activity (Sharma et al., 1981). Manganese-dependent enzymes fall into two categories: those which contain a tightly bound manganese and those which are loosely associated with their metal cofactor (McEuen, 1982). For the latter class of enzymes, which includes OT, stimulation of enzyme activity is often observed with the addition of exogenous manganese to the enzyme solution. In some cases, other similar metal cations may also effect enzyme activity. In light of the potential utility of manganese as a spectroscopic probe, particularly in EPR experiments (Campbell & Dwek, 1984), it is important to establish whether the metal cofactor in this enzyme plays an implicit role in catalysis or serves simply in a structural capacity.

A powerful tool for assessing the potential role of a metal ion in enzyme catalysis involves the parallel kinetic analyses of substrates and substrate analogs that differ only in the coordinating properties of the heteroatoms at or near the reacting center. This approach has been used in the presence of different metal ions to provide valuable information on the direct catalytic role of the cofactors in the hammerhead RNA self-cleavage reaction (Dahm & Uhlenbeck, 1991) and in carboxypeptidase-mediated amide hydrolysis (Mock et al., 1981). We recently reported that the incorporation of thioasparagine, in place of asparagine, in tripeptide substrates for OT afforded competent substrates for the glycosylation reaction (Imperiali et al., 1992). This key observation has prompted us to exploit the thioasparagine reactivity in an investigation of the role of metal ions in OT catalysis. Herein, we report the kinetic analysis of the interaction of Bz-Asn-Leu-Thr-NHMe (**1**) and Bz-Asn(γS)-Leu-Thr-NHMe (**2**) with OT in the presence of divalent manganese, iron, calcium, and magnesium cations.



1: X=O
2: X=S

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[1] Abbreviations: Amb, γ-aminobutyrine; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; Asn(γS), thioasparagine; Bz, benzoyl; DMSO, dimethyl sulfoxide; dpm, disintegrations per minute; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; Glc, glucose; GlcNAc, *N*-acetylglucosamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; Man, mannose; NP-40, Nonidet P-40; OT, oligosaccharyl transferase; PC, phosphatidylcholine; TLC, thin-layer chromatography; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine.

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MATERIALS AND METHODS

Buffers and Solutions. Buffer A contained 50 mM HEPES, pH 7.4, and 1.2% Triton X-100. Buffer B contained 50 mM HEPES, pH 7.4, 140 mM sucrose, 1% NP-40, and 0.5 mg/mL PC. Buffer C contained 50 mM HEPES, pH 7.4, 1 mM DTT, 500 mM NaCl, 140 mM sucrose, 0.1 mM AEBSF, 2 μg/mL leupeptin, and 4 μg/mL pepstatin. Buffer D contained 50 mM HEPES, pH 7.4, 1 mM DTT, 50 mM NaCl, 140 mM sucrose, 0.1 mM AEBSF, 2 μg/mL leupeptin, 4 μg/mL pepstatin, and 0.1% NP-40. Buffer E contained 50 mM HEPES, pH 7.4, 1 mM DTT, 500 mM NaCl, 140 mM sucrose, 1% NP-40, 1 mg/mL PC, 0.1 mM AEBSF, 2 μg/mL leupeptin, and 4 μg/mL pepstatin. Theoretical upper phase (TUP): 12:192:186:2.69 chloroform/methanol/ water/ 0.25 M $MgCl_2$ (Behrens & Tabora, 1978).

Peptide Synthesis. Peptides **1** and **2** were synthesized by standard solution-phase coupling methods. Bz-Asn(γS)-Leu-Thr-NHMe (**2**) was prepared as previously described by converting β-cyanoalanine to thioasparagine by sequential treatment with saturated ammonia in ethanol followed by hydrogen sulfide (Imperiali et al., 1992).

Glycolipid Synthesis. Dolichol-P-P-[^{3}H]GlcNAc-GlcNAc (^{3}H labeled at the C-6 position of the β-1,4-linked GlcNAc) was prepared as described previously (Imperiali & Zimmerman, 1990). Material of the desired specific activity for each experiment was obtained by appropriate dilution of high specific activity UDP-[^{3}H]GlcNAc (ca. 36 Ci/mmol, NEN) prior to the biosynthetic preparation of the lipid-linked disaccharide.

Glycopeptide Synthesis. Five samples of dolichol-P-P-GlcNAc-[^{3}H]GlcNAc (200 000 dpm, 0.011 Ci/mmol) were each dissolved in 20 μL of 5 mM Bz-Asn-Leu-Thr-NHMe in DMSO. Buffer B was added to each sample in 75-μL aliquots. The glycosylation reaction was initiated by the addition of 100 μL of solubilized porcine apoenzyme (see below) and 5 μL of 600 mM $MnCl_2$. After agitation for 4 h at room temperature, the five reactions were quenched together into 6 mL of 3:2:1 chloroform/methanol/4 mM $MgCl_2$. The aqueous layer was removed, and the organic layer was washed with 2 × 3 mL of TUP. The combined aqueous layers were washed with 1 mL of chloroform and then dried under a stream of nitrogen. The resulting residue was partially purified by gradient elution with acetonitrile from a SepPak cartridge (C-18, Millipore). The product was detected by monitoring for radioactivity, which typically eluted from the cartridge between 15% and 25% acetonitrile in water. Final purification was accomplished by reverse-phase HPLC (on a C-18 analytical column, 25 cm × 4.6 mm) with a 0.1% TFA/acetonitrile/water gradient from 15% to 20% acetonitrile over 25 min. Typically, 1 000 000 dpm dolichol-P-P-GlcNAc-[^{3}H]GlcNAc afforded 250 000 dpm pure Bz-Asn(GlcNAc-[^{3}H]GlcNAc)-Leu-Thr-NHMe (**3**).

A similar procedure was utilized for the preparation of Bz-Asn(γS)(GlcNAc-[^{3}H]GlcNAc)-Leu-Thr-NHMe (**4**). Dolichol-P-P-GlcNAc-[^{3}H]GlcNAc was dissolved in 5 μL of DMSO and 15 μL of a 10 mM DMSO solution of **2** and combined with 75 μL of buffer B and 100 μL of the solubilized porcine apoenzyme. The reaction was initiated by the addition of 5 μL of 600 mM $MnCl_2$ or 600 mM $Fe(NH_4)_2(SO_4)_2$. The reaction mixtures were agitated for 12 h at room temperature and then quenched and purified as described above. HPLC analysis indicated the preparation of one radiolabeled product (**4**) and several non-radiolabeled side products. Utilization of the two different divalent metals yielded identical HPLC chromatograms.

The syntheses of non-radiolabeled glycopeptides **3** and **4** were accomplished as described above. Dolichol-P-P-GlcNAc-GlcNAc was prepared enzymatically from UDP-GlcNAc and dolichol-P-P-GlcNAc by using protocols identical to those developed for the synthesis of radiolabeled material (Imperiali & Zimmerman, 1990). The progress of the reaction was estimated by monitoring a parallel reaction using radiolabeled UDP-GlcNAc at identical concentrations. Products were purified by HPLC, and elution times were identical to those of the corresponding radiolabeled analogs. The product identities were further verified by mass spectroscopy. Molecular weights for the two unlabeled glycosylated peptides, **3** and **4**, were determined by MALDI–time of flight mass spectroscopy using a 2,5-dihydroxybenzoic acid matrix. Calcd MW for **3**, 869.9 (MNa^+: 892.9); obsd MNa^+, 893.5. Calcd MW for **4**, 885.9 (MNa^+: 908.9); obsd MNa^+, 909.6. The purified glycopeptide **4** was also analyzed for purity by capillary zone electrophoresis. The results indicate that the sample obtained in the HPLC purification migrates as predominantly (94%) one peak.

The solution-state stabilities of **2** and **4** under the reaction conditions used throughout these experiments were assessed as follows. Four control experiments, A–D, were prepared by combining 5 μL of 600 mM $MnCl_2$, 5 μL of DMSO, and 155 μL of buffer A. Reaction mixtures for experiments A–C contained 15 μL of 10 mM **2** in DMSO, and that for experiment D contained 15 μL of 10 mM **1** in DMSO. Experiment A was initiated by the addition of 20 μL of solubilized enzyme. Experiment B was initiated by the addition of 20 μL of buffer A. Experiment C was prepared in the presence of 50 000 dpm dolichol-P-P-GlcNAc-[^{3}H]-GlcNAc and was initiated by the addition of 20 μL of solubilized enzyme. Experiment D was also initiated by the addition of 20 μL of solubilized enzyme. Reaction mixtures for experiments A–D were agitated for 12 h, quenched, and purified as described above. HPLC traces for all four reactions were obtained.

Mercuric Acetate Reactions. Pure, radiolabeled glycopeptides **3** and **4** (50 000 dpm each, 0.011 Ci/mmol) were dissolved in 20 μL of acetonitrile, and mercuric acetate (80 μL of a 1 mg/mL water solution) was added to each glycopeptide. The reaction mixtures were agitated overnight and filtered through microcentrifuge filters (5000 rpm × 40 min). Filtered samples were used immediately or stored at −80 °C until ready for HPLC analysis.

Raney Nickel Reactions. The glycopeptide (**3** or **4**, 50 000 dpm, 0.011 Ci/mmol) was dissolved in 150 μL of water, and excess activated Raney nickel in ethanol was added (20 μL of an approximately 1:1 Raney nickel/ethanol slurry). After 3 h of agitation, a second aliquot of Raney nickel (20 μL) was added and the reaction mixture was shaken for an additional 3 h. The reaction mixture was centrifuged briefly to settle the catalyst, and the supernatant was removed. The catalyst was washed 4 times with 200 μL of 20% acetonitrile in water. The supernatants were pooled, and any remaining catalyst was removed through centrifugation. The final supernatant was dried under a stream of nitrogen and redissolved in water for HPLC and TLC analysis.

HPLC Analysis. Mercuric acetate and Raney nickel reactions were examined by analytical HPLC. Fractions were collected over 1 min intervals and quantitated for ^{3}H content. Absorbances were monitored at 228 and 254 nm.

TLC Analysis. Raney nickel reaction mixtures were applied to TLC plates (250 μM, glass, EM Science) in 10 000 dpm fractions and dried under vacuum. The plate was developed 6 times in 65:35:4:4 chloroform/methanol/water/15 M ammonium hydroxide (Warren & Jeanloz, 1978). Compound **3** ($R_f \approx 0.6$), chitobiose (**5**, $R_f = 0.33$), and 2-acetamido-1,5-anhydro-4-*O*-(2-acetamido-2-deoxyglucopyranosyl)-2-deoxyglucitol (Hiraizumi et al., 1993) (**8**, $R_f = 0.53$) were included on the same TLC plate for comparison. The plate was analyzed for ^{3}H content by removing the silica in 0.5-cm portions. Each sample was suspended in 1 mL of water and counted (dpm) in 6 mL of Ecolite (ICN). Nonradiolabeled lanes were visualized by heating the TLC plate after immersion in a 5% H_2SO_4 methanolic solution.

Saccharomyces cerevisiae Oligosaccharyl Transferase. A solubilized preparation of the yeast enzyme was prepared as previously described (Sherman & Spatola, 1990). Prior to assay, the crude enzyme was diluted 7-fold with buffer A and the $MnCl_2$ concentration was brought to 10 mM. A 200 000 dpm aliquot of dolichol-P-P-GlcNAc-[^{3}H]GlcNAc (specific activity, 36.5 Ci/mmol) was brought to dryness in a microcentrifuge tube. The lipid-linked donor was then redissolved in 10 μL of DMSO, 10 μL of 20 mM Bz-Asn-(γS)-Leu-Thr-NHMe (**2**) in DMSO, and 30 μL of buffer A. The assay was initiated by the addition of 150 μL of the diluted enzyme. Four time points were obtained by removing 40-μL aliquots at 2-min intervals. Each aliquot was quenched in 1.2 mL of 3:2:1 chloroform/methanol/4 mM $MgCl_2$. The aqueous layer was removed, and the organic layer was washed twice with 0.6 mL of TUP. The aqueous layers were combined and counted in 5 mL of Ecolite.

In order to determine whether **2** is a competitive inhibitor for OT activity, three samples of dolichol-P-P-[^{3}H]GlcNAc-GlcNAc (50 000 dpm, 36.5 Ci/mmol) were each redissolved in 10 μL of 1 mM Bz-Asn-Leu-Thr-NHMe in DMSO and 155 μL of buffer B (with 10 mM $MnCl_2$). An additional 10 μL of DMSO or of a 1 mM or a 10 mM DMSO solution of Bz-Asn(γS)-Leu-Thr-NHMe was added to each of the three reaction mixtures to bring the final concentration of **2** to 0, 50, and 500 μM, respectively. Each assay was initiated by the addition of 20 μL of undiluted, solubilized yeast OT. Time points were obtained as described above.

Porcine Liver Oligosaccharyl Transferase. Crude porcine liver microsomes were prepared from fresh pig liver (Imperiali & Shannon, 1991) and solubilized by a modified version of the procedure published for the solubilization of canine pancreatic microsomes (Kelleher et al., 1992). Crude liver microsomes (30 mL, 30%, v/v, glycerol suspension) were diluted to 150 mL with buffer C. This mixture was incubated at 0 °C for 20 min and centrifuged for 60 min at 2.5×10^5 g. The pellets were resuspended in 150 mL of buffer D. This mixture was incubated for 20 min at 0 °C and then centrifuged for 60 min at 2.5×10^5 g. The pellets were resuspended in 50 mL of buffer E and centrifuged, following a 20-min incubation, for 60 min at 1.4×10^5 g. The supernatant was divided into two portions (25 and 20 mL) and stored at −80 °C.

Apoenzyme for metal reconstitution experiments was prepared as follows. The solubilized porcine microsomes (25 mL) were gently thawed and treated twice with Chelex resin (Bio-Rad) at 4 °C for 5 min each. A brief centrifugation (2 min at 2000 rpm) facilitated the separation of the resin from the supernatant. The solution was then brought to a final concentration of 5 mM EDTA and incubated for 5 min at 4 °C. This solution was dialyzed twice against 500 mL of buffer E for 30 min each time. The apoenzyme could be stored at −80 °C for several months without loss of activity. Regeneration of activity was obtained by the addition of exogenous $MnCl_2$, $MgCl_2$, $CaCl_2$, or $Fe(NH_4)_2(SO_4)_2$ at concentrations in excess of 2 mM. Other divalent metal salts, including $ZnCl_2$, $NiCl_2$, $CdCl_2$, and $CoCl_2$, failed to restore activity. A 2-fold dilution of this enzyme was used for all experiments. This dilution resulted in 56% transfer of GlcNAc-[^{3}H]GlcNAc to yield **3** in 8 min with a peptide concentration of 1 mM and 200 000 dpm dolichol-P-P-GlcNAc-[^{3}H]-GlcNAc (specific activity, 36.5 Ci/mmol).

Determination of Kinetic Constants for **1** *and* **2**. In a typical experiment, six aliquots of dolichol-P-P-GlcNAc-[^{3}H]-GlcNAc (200 000 dpm; specific activity, 36.5 Ci/mmol) were blown dry under a stream of nitrogen. A DMSO solution of Bz-Asn(γS)-Leu-Thr-NHMe was added in 20-μL aliquots to each to yield final peptide concentrations of 2 mM, 1 mM, 500 μM, 250 μM, and 125 μM. A blank was prepared in the same manner using 20 μL of DMSO. Buffer A was added to each tube in 75-μL aliquots. The solubilized porcine apoenzyme (700 μL) was combined with 35 μL of a 400 mM solution of $MnCl_2$, $MgCl_2$, $CaCl_2$, or $Fe(NH_4)_2(SO_4)_2$, and 105 μL of this solution was used to initiate each assay. Aliquots (40 μL each) were removed after appropriate intervals and were treated as described above. The organic layers were dried, and the remaining proteinaceous residue was redissolved in 200 μL of Solvable (NEN), agitated for 30 min, and counted in 6 mL of Formula 989 (NEN). The amount of radioactivity present in the aqueous extracts was normalized against the total radioactivity (aqueous and organic) for each sample. Each set of kinetic values (K_M and V_{max}) was determined separately from the same batch of solubilized apoenzyme, and peptide concentrations were varied as needed.

RESULTS

The kinetic constants for compounds **1** and **2** have previously been determined with porcine liver oligosaccharyl transferase in the presence of Mn^{2+} (Imperiali et al., 1992). Compound **1** is also an excellent substrate for yeast OT (Imperiali et al., 1994); however, the thioamide **2** is not glycosylated and behaves as a competitive inhibitor with an approximate K_i of 100 μM. Because of the poor substrate behavior of **2** with yeast OT, all further studies were conducted with the porcine enzyme.

The porcine liver apoenzyme was partially purified and detergent solubilized. The removal of divalent metals was confirmed by the complete absence of OT activity in an assay. After solubilization and in the presence of sucrose and PC, the enzyme was stable for several months at −80 °C. Complete activity could be restored by the addition of $MnCl_2$, and partial activity could be restored by the addition of $MgCl_2$, $CaCl_2$, or $Fe(NH_4)_2(SO_4)_2$. In each case, relatively high metal concentrations were employed to ensure that maximum enzymatic activity was observed. Treatment with $ZnCl_2$, $NiCl_2$, $CdCl_2$, and $CoCl_2$ failed to restore activity under analogous conditions.

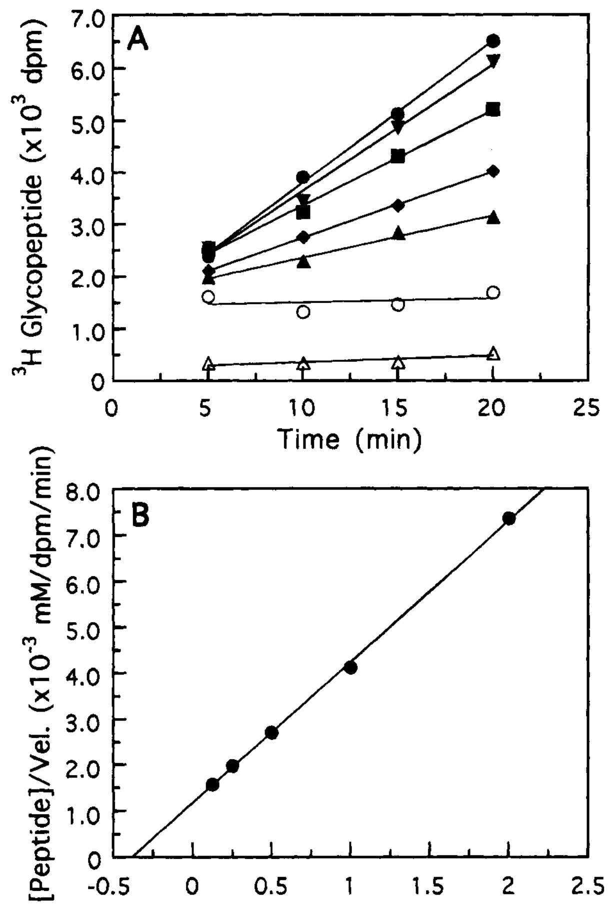

FIGURE 1: Kinetic analysis of Bz-Asn(γS)-Leu-Thr-NHMe (**2**) with $MnCl_2$. (A) Relative rates of glycosylation with increasing concentrations of **2** (○, 0 mM; ▲, 0.125 mM; ◆, 0.25 mM; ■, 0.5 mM; ▼, 1.0 mM; ●, 2.0 mM) in the presence of 10.5 mM $MnCl_2$. Apoenzyme was also assayed without added metal cations (△, 0 mM **2** and 0.5 mM Bz-Asn-Leu-Thr-NHMe (**1**)). (B) Hanes plot of **2** with $MnCl_2$.

Kinetic analyses on **1** and **2** were performed in the presence of four different divalent metal ions: Mn^{2+}, Fe^{2+}, Mg^{2+}, and Ca^{2+}. These metals were chosen because they produce significant rates of turnover with **1** and they represent a wide range of ionic radii (0.64–0.99 Å), as well as different affinities for sulfur ($Fe^{2+} > Mn^{2+} \gg Mg^{2+} \approx Ca^{2+}$). Each assay was run at a final metal concentration of 10.5 mM, which was well within the range of maximum activity for each of the four metals. Since the kinetic experiments did not control for Fe^{2+} oxidation, the possible effects of Fe^{3+} were also considered. Control experiments indicate that enzyme activity is not reconstituted with Fe^{3+}. Furthermore, in the presence of 10 mM Fe^{2+}, concentrations of Fe^{3+} below 0.5 mM did not inhibit enzyme activity. Partial inhibition of OT activity due to the oxidation of Fe^{2+} to Fe^{3+} was observed after 6–8 h of incubation in the preparative product synthesis; this inhibition would not be significant on the 20-min time scale of the kinetic experiments.

Each set of kinetic data was obtained from the same stock of apoenzyme to allow for comparison between data sets. Kinetic constants were calculated on the basis of data obtained from a Hanes plot (*S*/*V* vs *S*, where *S* equals the peptide concentration and *V* is the relative maximum velocity observed at each concentration) (Cornish-Bowden, 1979).

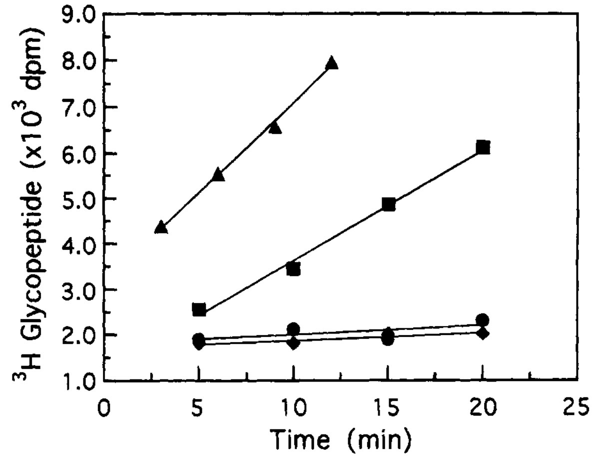

FIGURE 2: Relative rates of Bz-Asn(γS)-Leu-Thr-NHMe (**2**) with different divalent metal ions. Peptide concentration was 1.0 mM. Final metal concentrations were 10.5 mM (▲, $Fe(NH_4)_2(SO_4)_2$; ■, $MnCl_2$; ◆, $MgCl_2$; ●, $CaCl_2$).

Table 1: Kinetic Parameters for **1** and **2** in the Presence of Various Divalent Metal Cations

	Bz-Asn-Leu-Thr-NHMe (**1**)		Bz-Asn(γS)-Leu-Thr-NHMe (**2**)	
metal ion	apparent K_M (μM)	V_{max} (dpm/min)	apparent K_M (μM)	V_{max} (dpm/min)
Mn^{2+}	380	4550	380	326
Fe^{2+}	650	2120	680	703
Ca^{2+}	1100	4800	N/A	no rate
Mg^{2+}	2500	2930	>5000	<100

Figure 1 shows representative kinetic data for manganese with peptide **2** (see supporting information for remaining kinetic data). Background rates for each metal were assessed in the absence of peptide substrate. These rates were very low and may be due either to glycosylation of endogenous proteins or to metal-mediated breakdown of the glycosyl donor. Control experiments indicated that the background rates are suppressed in the presence of the tripeptide substrates; therefore, the rate data were used directly for calculation of the kinetic constants without correction. The Michaelis–Menten constants for both **1** and **2**, with OT and each metal ion, are listed in Table 1 for comparison. These results represent constants obtained from a typical set of experiments.

The concentration of dolichol-P-P-GlcNAc-[^{3}H]GlcNAc was maintained at 12.6 nM for each kinetic experiment. This concentration is well below the reported K_M of 1.2 μM for the lipid-linked substrate (Sharma et al., 1981). The possibility that this K_M could vary when the enzyme was reconstituted with the various metals was considered. However, when the dolichol-P-P-GlcNAc-[^{3}H]GlcNAc concentration was increased to 41.3 μM (above K_M) for the glycopeptide synthesis, similar trends in relative velocities were observed.

Oligosaccharyl transferase activity, when regenerated with manganese, produces the optimum kinetic parameters with compound **1**. The K_M values obtained for **1** and **2** in the presence of Mn^{2+} were found to be similar (380 μM each). However, the V_{max} for the sulfur-containing compound **2** was approximately 1 order of magnitude lower than that for the corresponding oxygen-containing peptide **1**. When enzymatic activity was restored with Fe^{2+}, **1** and **2** still exhibited similar binding constants (650 and 680 μM, respectively).

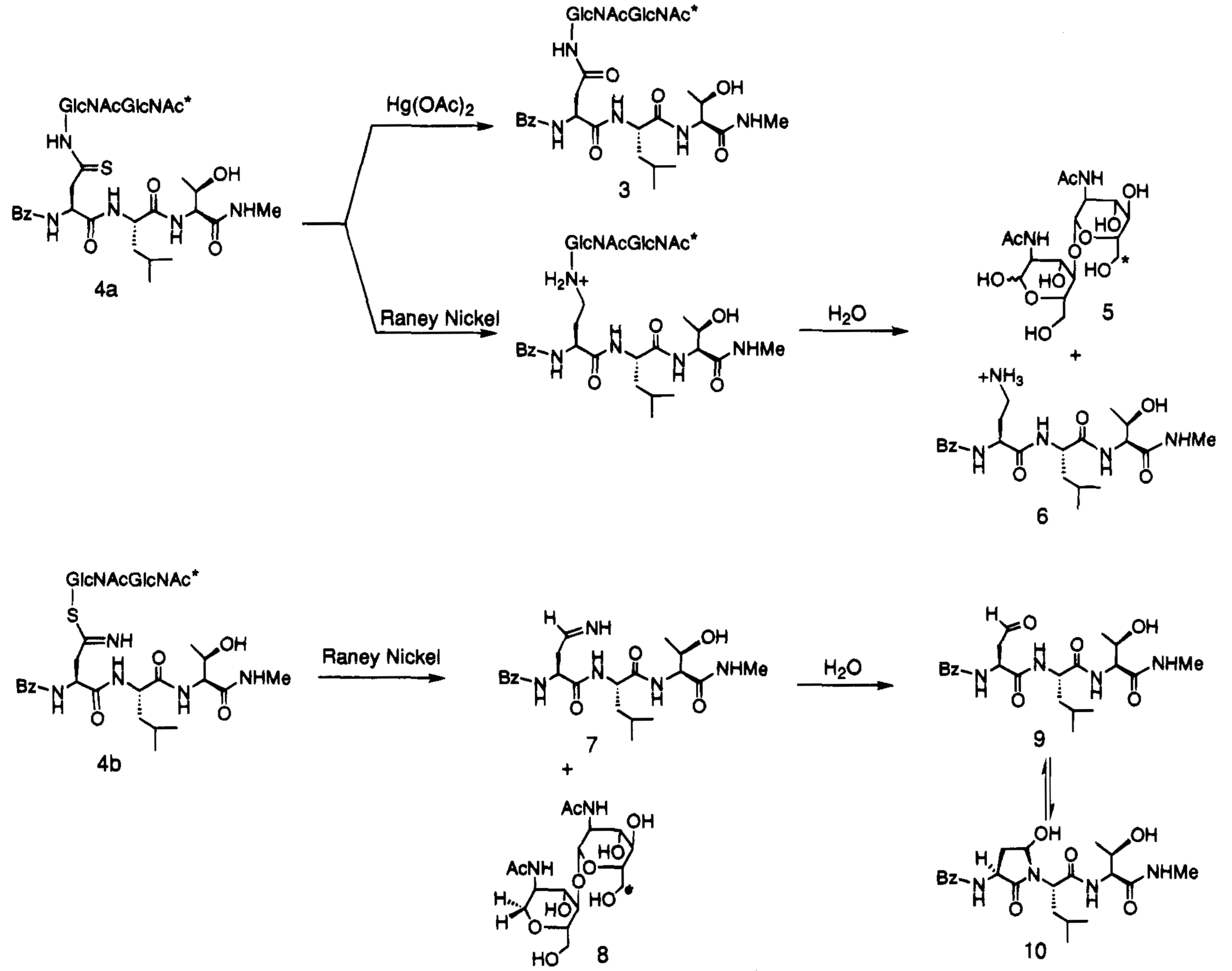

FIGURE 3: Chemical analysis of glycopeptide **4**.

Notably, thioamide **2** was turned over at a rate which was only three times slower than that for **1**, and in fact, the relative maximum velocity for **2** doubled when manganese was replaced with iron. The trend in the kinetic parameters for calcium and magnesium is quite distinct. While the amide substrate, **1**, is turned over with reasonable efficiency in the presence of each of these metals, the thioasparagine peptide **2** is not glycosylated at all in the presence of calcium and is very poorly glycosylated in the presence of magnesium (Figure 2).

Several experiments were designed and implemented to confirm the identities of glycopeptides **3** and **4** (Figure 3). The enzymatic synthesis of **3** yielded an HPLC chromatogram with only two peaks, the starting material **1** and the product **3**. The radiolabeled product eluted approximately 2 min earlier than the starting material. The retention time and the presence of the tritium label were consistent with the formation of a glycosylated product. Mass spectral analysis confirmed the identity of the product peak.

For the turnover of peptide **2**, products resulting from glycosylation at sulfur or nitrogen were considered. For clarity, the isolated material is designated as **4**, and the two potential products are designated as **4a** for nitrogen glycosylation and **4b** for sulfur glycosylation. Enzymatic synthesis of **4** produced an HPLC chromatogram which contained the desired glycopeptide as well as several small side products and starting material. The radioactivity quantitatively eluted with the glycopeptide peak approximately 4 min earlier than the corresponding peptide. These results were independent of the divalent metal ion (Mn^{2+} or Fe^{2+}) used to prepare the glycopeptide. Control experiments, in the absence of enzyme, indicated that the observed side products result from the normal decomposition of **2**; this breakdown is most likely due to succinimide formation with the amide nitrogen of the adjacent amino acid (Brennan & Clarke, 1995). The rate of decomposition was insignificant on the time scale of the kinetic experiments and was only observed during the longer glycopeptide synthesis and isolation procedures. Mass spectral analysis of the purified product afforded the correct molecular weight but could not be used to distinguish between the two possible products (**4a** and **4b**), which have the same molecular formula. Capillary zone electrophoresis of **4** yielded one major peak (94%).

Since **4** was only available in microgram quantities, the glycosylation regiochemistry had to be assessed through the use of analytical methods which were amenable to HPLC and TLC characterization. The chemical transformations that were investigated are summarized in Figure 3. Mercuric acetate has previously been shown to convert thioamides to their corresponding oxoamides in organic solvents (Davies & Mortlock, 1991, 1993); model reactions on *N*-ethylthioacetamide indicated that mercuric acetate would effect the

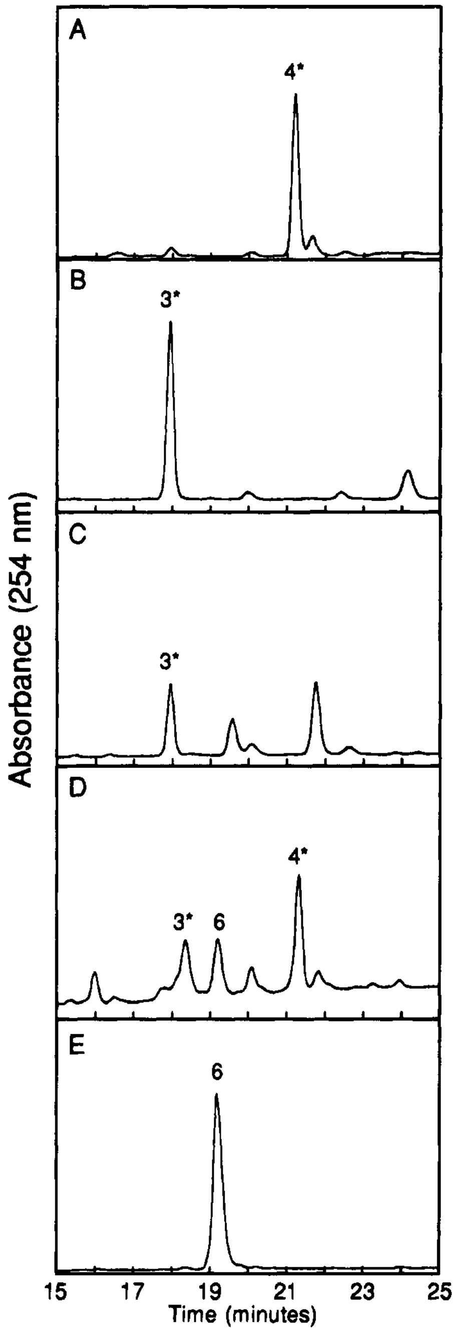

FIGURE 4: Reverse-phase HPLC chromatograms obtained on a C-18 analytical column, 25 cm × 4.6 mm, with a 0.1% TFA/acetonitrile/water gradient from 10% to 30% acetonitrile over 30 min. Aliquots were counted for radioactivity at 1 min intervals. (A) Bz-Asn(γS)-[GlcNAcGlcNAc]-Leu-Thr-NHMe (**4**). (B) Bz-Asn[GlcNAcGlcNAc]-Leu-Thr-NHMe (**3**). (C) Treatment of Bz-Asn(γS)[GlcNAcGlcNAc]-Leu-Thr-NHMe (**4**) with mercuric acetate. (D) Treatment of Bz-Asn(γS)[GlcNAcGlcNAc]-Leu-Thr-NHMe (**4**) with Raney nickel. (E) Bz-Amb-Leu-Thr-NHMe (authentic sample). Radiolabeled compounds are designated with an asterisk (*).

same conversion in water. As determined by HPLC analysis (Figure 4A–C), treatment with mercuric acetate converted compound **4** to glycopeptide **3** (Figure 4C). When the radioactivity was quantified, 71% of the tritium label was found to coelute with the product glycopeptide (**3**). The remaining 29% eluted in a broad peak which was not visible at 228 or 254 nm, suggesting the presence of a carbohydrate fragment. As a control, compound **3** was also treated with mercuric acetate. In this case, no reaction occurred and the radiolabel quantitatively eluted with **3**. These results are consistent with at least 71% of the glycosylation occurring on the nitrogen of the thioasparagine side chain (**4a**). The reactivity of **4b** in the presence of mercuric acetate was not predicted.

Raney nickel reductively cleaves carbon–sulfur bonds in both aqueous and organic media. As illustrated in Figure 3, treatment of **4a** with Raney nickel would be expected to afford a labile product which, in the presence of water, would hydrolyze to chitobiose (**5**) and Bz-Amb-Leu-Thr-NHMe (**6**). HPLC analysis (Figure 4D,E) of the Raney nickel products indicated the presence of **6** when compared to an authentic sample (Imperiali et al., 1992). Furthermore, TLC analysis indicated that 40–50% of the radiolabel was converted from **4** to chitobiose (**5**). In addition, the Raney nickel treatment resulted in the production of 15–25% glycopeptide **3**. The desulfurization of **4b** by Raney nickel would be expected to yield two products (**7** and **8**, Figure 3). The imine, **7**, if formed, should hydrolyze to the aldehyde **9**, which is in equilibrium with the cyclic aminal **10**. The second product would be 1-deoxychitobiose (**8**) (Hiraizumi et al., 1993). Both **8** and **10** were synthesized for comparison. Compound **10** was not observed by HPLC. In the TLC analysis compound **8** was not observed; however, an unidentified radiolabeled product was present which accounted for 25–35% of the radioactivity.

The possibility that the product **4b** was labile under the reaction conditions was also considered. This reaction course would be manifested by the appearance of increased levels of peptide **1** or the corresponding glycopeptide **3**. Hydrolysis of **4** to afford **1** and **3** was not observed.

DISCUSSION

The reconstitution experiments with oligosaccharyl transferase indicate that the enzyme can function in the presence of any of several metal ions including Mn^{2+}, Fe^{2+}, Ca^{2+}, and Mg^{2+}. These metal ions share a common feature in that each can adopt an octahedral coordination geometry. Divalent cations that show preferences for other coordination geometries, including Ni^{2+}, Cu^{2+}, Cd^{2+}, and Zn^{2+}, fail to activate the enzyme, and at high concentrations also competitively inhibit activity in the presence of manganese.

In order to gain insight on the specific role of the metal cation in catalysis, we have investigated the influence of the metal cofactor on catalytic turnover for two substrates, **1** and **2**, which differ only in the replacement of an oxygen atom with a sulfur at the reaction center. The kinetic constants calculated for each peptide in the presence of four different divalent metal cations followed distinct trends (Table 1). The metal ion substitution studies reveal that the asparagine-containing tripeptide is efficiently turned over in the presence of several metal cations (Mn^{2+}, Fe^{2+}, Mg^{2+}, and Ca^{2+}). For the thioasparagine peptide (**2**), the thiophilicity of the metal cation directly correlates with the efficiency of turnover. Both iron and manganese resulted in active complexes, and iron, the most thiophilic metal, yielded the highest relative rates. In contrast, when the enzyme is reconstituted with the oxophilic cations, magnesium and calcium, the thioasparagine peptide is very poorly glycosylated. The kinetic constants also appear to be correlated with the size of the metal cation. With peptide **1**, iron and magnesium (ionic radii of 0.66 and 0.64 Å, respectively) produced the lowest turnover rates and calcium and manganese (0.99 and 0.80 Å, respectively) produced the highest rates. A different effect was observed with peptide **2**, and this may be due to the fact that sulfur substitution imposes its own steric perturbations. Both the heteroatom covalent radius and the carbon–heteroatom bond length increase by about 25% on changing from oxygen to sulfur (Sherman & Spatola, 1990). In the case of calcium and peptide **2**, both binding and turnover were undetectable, and this may be due to both steric effects between the bulky sulfur and the calcium ion and the low thiophilicity of the metal.

In general, amide functional groups can undergo alkylation on the nitrogen or the oxygen to afford amide or imidate products, respectively. In solution, the relative distributions of these two products vary depending on the nature of the electrophile and the reaction conditions (Challis & Challis, 1970). In model systems, electrophilic attack on thioamides occurs preferentially at sulfur (Walter & Voss, 1970). In the asparagine-linked glycosylation process it is clear that the reaction is predominantly localized at nitrogen. This regiochemical control is important because the corresponding *O*-alkylated imidate products would be too labile to be viable in a physiological context. In these experiments it was imperative to establish whether the regiochemical control with the unnatural thioamide substrate paralleled that of the amide substrate. A detailed analysis of the glycosylated thioasparagine-containing peptide did not indicate the presence of sulfur alkylation products, as would be manifested either by the production of a labile product that spontaneously hydrolyzed to the corresponding asparagine-containing tripeptide or by formation of a stable product that could be converted to known authentic samples *via* Raney nickel treatment. In fact, at least 70% of the glycosylated species was directly identified as the N-linked glycopeptide. The identity of the remaining 30% has yet to be confirmed.

We have recently provided evidence that the fidelity of asparagine-linked glycosylation may be related to the ability of the peptide substrates to adopt an Asx-turn conformation (Imperiali et al., 1994). The hydrogen-bonding network seen in this motif, along with participation of the enzyme, is thought to direct the modification to the nitrogen of the bidentate functional group in the natural substrates. Although thioamides preferentially undergo sulfur alkylation in model systems (Walter & Voss, 1970), the observation that enzyme-catalyzed thioamide glycosylation proceeds predominantly or exclusively at the nitrogen is consistent with the environment of the functional group playing an important role in targeting the alkylation to the correct heteroatom despite the inherent chemical tendencies of the system.

In enzyme-catalyzed glycosylation of asparagine, the distinct preference for manganese over magnesium may argue against a specific role for the metal cation in coordination to the pyrophosphoryl moiety of the lipid-linked carbohydrate substrate (Frausto da Silva & Williams, 1991). The comparative studies on asparagine- and thioasparagine-containing tripeptides described in this paper implicate the metal cofactor in a role that places it proximal to the peptide binding site. We are now positioned to further investigate the divalent metal cation and catalysis by using the manganese center as a spectroscopic probe to evaluate the interactions of the enzyme with asparagine and thioasparagine substrates. This information will be important in future considerations of the mechanism of asparagine-linked glycosylation.

SUPPORTING INFORMATION AVAILABLE

Description of the synthetic procedures and spectroscopic data for compounds **5**, **8**, **9**, and **10**; kinetic results (graphs) for compounds **1** and **2** with Mn^{2+}, Fe^{2+}, Mg^{2+}, and Ca^{2+} (15 pages). Ordering information is given on any current masthead page.

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