

# Kinetic Analysis of NodST Sulfotransferase Using an Electrospray Ionization Mass Spectrometry Assay<sup>†</sup>

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**ABSTRACT:** A novel and efficient enzyme kinetics assay using electrospray ionization mass spectrometry was developed and applied to the bacterial carbohydrate sulfotransferase (NodST). NodST catalyzes the sulfuryl group transfer from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to chitobiose, generating 3'-phosphoadenosine 5'-phosphate (PAP) and chitobiose-6-OSO<sub>3</sub><sup>-</sup> as products. Traditional spectrophotometric assays are not applicable to the NodST system since no shift in absorption accompanies sulfuryl group transfer. Alternative assays have employed thin-layer chromatography, but this procedure is time-consuming and requires radioactive materials. The ESI-MS assay presented herein requires no chromophoric substrate or product, and the analysis time is very short. The ESI-MS assay is used to determine NodST kinetic parameters, including  $K_M$ ,  $V_{max}$ , and  $K_i$  (for PAP). In addition, the mode of inhibition for PAP was rapidly determined. The results were in excellent agreement with those obtained from previous assays, verifying the accuracy and reliability of the ESI-MS assay. This unique technique is currently being used to investigate the enzymatic mechanism of NodST and to identify sulfotransferase inhibitors.

Sulfated biomolecules regulate a diverse array of specific cellular communication events in both normal and pathological processes (1, 2), such as acute and chronic inflammation (3, 4), cancer metastasis (5), microbial pathogenesis (2), and hormone regulation (6). The participation of these bioconjugates in a variety of disease states has triggered interest in the enzyme family that installs the sulfate group, sulfotransferases. Sulfotransferases catalyze sulfuryl group (SO<sub>3</sub><sup>-</sup>) transfer from 3'-phosphoadenosine 5'-phosphosulfate (PAPS)<sup>1</sup> to an acceptor hydroxyl (or amino) group on a protein (7, 8), carbohydrate (1, 9), or a small molecule (10, 11). They play an important role in modulating normal and pathogenic processes and are now considered as potential therapeutic targets (1, 2).

Among the broad family of sulfotransferases, the human GlcNAc-6-*O*-sulfotransferases [e.g., CHST2 (12) and HEC-GlcNAc6ST (3, 4)] have become the focus of intense interest

due to their role in the biosynthesis of the L-selectin ligands that participate in both normal and pathological inflammatory responses (13–15). Unfortunately, the human GlcNAc-6-*O*-sulfotransferases are membrane-bound, and difficult to express at high levels. Therefore, in our efforts to develop small molecule inhibitors (16), we have initially focused our attention on a functionally related sulfotransferase, the GlcNAc-6-*O*-carbohydrate sulfotransferase NodST from the nitrogen-fixing bacterium *Rhizobium meliloti* (17). This enzyme acts as a host-specific nodulation switch by catalyzing the transfer of a sulfuryl group from PAPS to the 6-hydroxyl group of the reducing terminal GlcNAc residue of a lipochitooligosaccharide (18) (1, Scheme 1). The resulting sulfated lipochitooligosaccharide (2), or “nod factor”, is critical for root nodulation and bacterial infection (19). NodST can also utilize the simple disaccharide chitobiose (3) as a substrate, giving chitobiose-6-OSO<sub>3</sub><sup>-</sup> (4) as the product (20, 21) (Scheme 2). The assay described herein is a facile and broadly applicable kinetics assay for sulfotransferases using mass spectrometry and is used to characterize the kinetic parameters of the NodST sulfotransferase. NodST can be generated in large quantities via bacterial overexpression (22) and shares similar GlcNAc-6-*O*-sulfotransferase activity with the mammalian enzymes of therapeutic interest, making this enzyme an ideal model sulfotransferase for our preliminary studies. Insights gained from our work with NodST will facilitate later research with the mammalian enzymes.

The mechanistic investigation of the bisubstrate (chitobiose and PAPS) enzymatic reaction of NodST (20, 21) is critical for its optimization as a sulfotransferase model enzyme system. Enzyme kinetic parameters such as the Michaelis–

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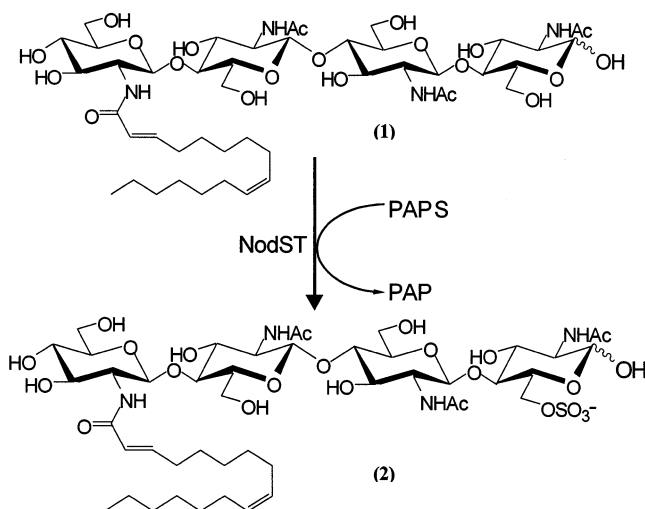
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<sup>1</sup> Abbreviations: CHST, carbohydrate sulfotransferase; ESI-MS, electrospray ionization mass spectrometry; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HEC, high endothelial cell; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PAP, 3'-phosphoadenosine 5'-phosphate; SIM, selected ion monitoring; Ni-NTA, nickel-nitrilotriacetic acid; TLC, thin-layer chromatography; Tris, tris-(hydroxymethyl)aminomethane; ΔD<sub>1</sub>-6S, α-ΔUA-[1→3]-GalNAc-6S.

Scheme 1: NodST Catalyzes the Sulfation of a Lipochitooligosaccharide (1)



Menten constant  $K_M$  and the maximum velocity  $V_{max}$  are usually determined by spectrophotometric methods, if the reactants undergo a detectable shift in absorption or fluorescence at a characteristic wavelength during the reaction. Unfortunately, the substrates and products of the enzymatic reactions catalyzed by some enzymes, including NodST, do not have such a shift in absorption that is needed to perform spectrophotometric methods. Although various strategies have been used to solve this problem, such as the synthesis of artificial chromophogenic or fluorogenic substrates (23–26), these strategies cannot usually be employed for group transfer reactions and such artificial substrates have not been designed for sulfotransferases yet. Recently, Bertozzi and co-workers have employed a radiolabel transfer assay (16) for sulfotransferases that involves the separation of the product,  $^{35}\text{S}$ -labeled chitobiose-6- $\text{OSO}_3^-$ , from the excess substrate,  $^{35}\text{S}$ -labeled PAPS, on a silica gel TLC plate followed by quantification via phosphorimaging. Using the TLC assay, a  $K_M$  value of  $4.3 \pm 0.3 \mu\text{M}$  was obtained for the substrate PAPS and a  $K_i$  value of the product PAP with respect to PAPS was determined to be  $1.36 \pm 0.08 \mu\text{M}$  (16). However, a  $K_M$  value for the other substrate, chitobiose, could not be determined using this method.

Recently, soft ionization methods such as electrospray ionization (ESI) and fast atom bombardment (FAB) mass spectrometry have been demonstrated to be complementary to conventional spectrophotometric methods for enzyme kinetics studies (27–30). We have developed a novel

electrospray ionization mass spectrometry (ESI-MS) assay using ion trap mass spectrometry that does not require chromophogenic substrates or products (31). Because a single-point normalization factor is used, a calibration curve is not required and the total analysis time is approximately the same as that of the standard spectrophotometric assay. This method can be applied to any enzyme system if an internal standard with structure and ionization efficiency comparable to those of the product or substrate is available. An additional advantage is that substrate and product quantities can be simultaneously analyzed during the course of the reaction. Thus, the ESI-MS assay is an efficient method for determining enzyme kinetic parameters, as demonstrated here with NodST.

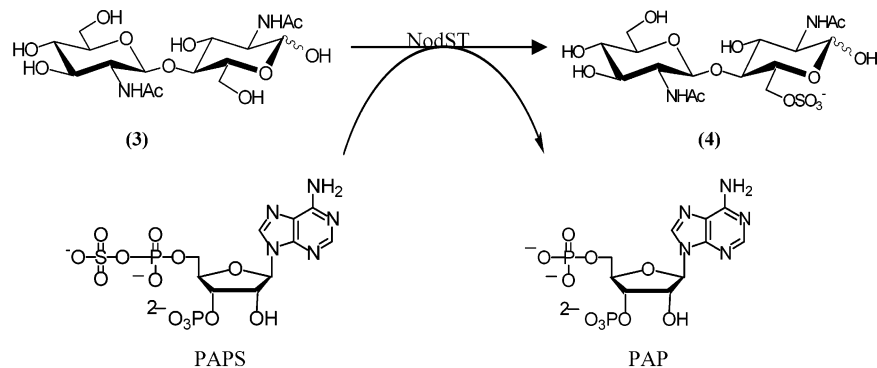
The ESI-MS assay was initially applied to the NodST bisubstrate enzymatic system, in which NodST catalyzes the transfer of a sulfuryl group from the sulfate donor PAPS to the sulfate acceptor chitobiose, yielding PAP and chitobiose-6- $\text{OSO}_3^-$  as products (Scheme 2). Utilizing the internal standard, a chondroitin disaccharide called  $\alpha$ - $\Delta\text{UUA}$ -[1 $\rightarrow$ 3]-GalNAc-6S ( $\Delta\text{D}_1$ -6S) (Scheme 2), a single-point normalization factor between the product and the internal standard was obtained. The kinetics results of the ESI-MS assay were in excellent agreement with previously published values. This assay is currently being used to explore the catalytic mechanism of our model enzyme NodST and is also being adapted to mammalian carbohydrate sulfotransferases of therapeutic interest.

## EXPERIMENTAL PROCEDURES

### General Materials and Methods

All chemical reagents were obtained from commercial suppliers and used without further purification. Bovine serum albumin (BSA), NodST control, and chitobiose were purchased from Calbiochem (San Diego, CA). The Ni-NTA agarose (25 mL) was purchased from QIAGEN Inc. (Valencia, CA). All the other substrates, internal standards, and inhibitors were purchased from Sigma Co. (St. Louis, MO), including 3'-phosphoadenosine 5'-phosphosulfate (PAPS),  $\alpha$ - $\Delta\text{UUA}$ -[1 $\rightarrow$ 3]-GalNAc-6S ( $\Delta\text{D}_1$ -6S), and 3'-phosphoadenosine 5'-phosphate (PAP).

Water used for the biochemical procedures was doubly distilled and deionized using a Milli-Q system (Millipore). *Escherichia coli* was purchased from NEB, and the pET-24d-NodH vector was cloned as previously described (22). NodST containing a His<sub>6</sub> tag was expressed from *E. coli* cells

Scheme 2: NodST Catalyzes the Sulfation of Chitobiose (3), Generating PAP and Chitobiose-6- $\text{OSO}_3^-$  (4) as Products

and purified using nickel–nitrilotriacetic acid (Ni–NTA) metal affinity chromatography as previously described (22). The purification procedures were performed at 4 °C to prevent the protein from denaturing. The NodST solution was dialyzed against 100 mM Tris (pH 8.0) containing 20 mM  $\beta$ -mercaptoethanol and 10% glycerol to remove  $\text{Na}^+$  before use in the mass spectrometric assay. The NodST concentration was determined using the standard Lowry assay. Agarose gel electrophoresis was performed using standard procedures. The TLC assay was performed to check the activity of expressed NodST as previously described (16). The activity of the expressed NodST was determined to be 50 nM unit<sup>-1</sup> ( $k_{\text{cat}} = 19.8 \text{ min}^{-1}$ ). This is compared to the literature  $k_{\text{cat}}$  value of 23.6 min<sup>-1</sup> (22) and a calculated NodST activity of 42 nM unit<sup>-1</sup>. All the mass spectrometric kinetics assays were performed at 22 °C in 10 mM  $\text{NH}_4\text{-OAc}$  (pH 8.0) (buffer A).

### Mass Spectrometry

A Finnigan LCQ ion trap mass spectrometer equipped with an ESI source and an HPLC pump (Thermo-Finnigan, San Jose, CA) was used. The capillary temperature and the spray voltage were kept at 200 °C and 3.2 kV, respectively. Approximately 40  $\mu\text{L}$  of each sample solution was delivered via the LC pump at a flow rate of 20  $\mu\text{L}/\text{min}$ . The product ion ( $m/z$  503) and the internal standard ion ( $m/z$  458) were monitored in the negative ion mode using selected ion monitoring (SIM). The signals for the ions of interest were optimized by using the automatic tuning option on the instrument. The optimized conditions were then applied in subsequent experiments. When the signal intensity for one sample decreased from approximately  $1 \times 10^6$  detector counts per scan to  $1 \times 10^4$  detector counts per scan, indicating the consumption of the former injection, the next sample was injected.

The Qual Browser program was used to analyze the data from the chromatogram and spectrum list. The chromatogram of the Qual Browser program was used to monitor the processing of samples versus time, with each peak representing a different sample that was monitored. An average of 60 ( $12 \times 5$ ) scans was taken to obtain a spectrum list for each sample, which provides the absolute intensities for the monitored ions along with relative abundance. The sums of the intensities within 0.8 mass unit around the center of product ion peak and internal standard ion peak were used to determine their intensity ratio ( $I_{\text{P}}/I_{\text{S}}$ ). This ratio was subsequently used to obtain a single-point normalization factor, quantify the amount of product, and calculate the initial velocity in the enzymatic reaction.

### Enzyme Kinetics

**Single-Point Normalization Factor.** A stock solution of NodST (1.8  $\mu\text{M}$ ) (NodST stock 1) was prepared in buffer A. Stock solutions of chitobiose (0.5 and 5.0 mM), PAPS (12.5 and 125  $\mu\text{M}$ ), and  $\alpha$ - $\Delta\text{UA}$ -[1 $\rightarrow$ 3]-GalNAc-6S ( $\Delta\text{D}_i$ -6S) (250  $\mu\text{M}$ ) were prepared in buffer A. A volume of 110  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$  of a PAPS stock solution (125  $\mu\text{M}$ ), and 40  $\mu\text{L}$  of a chitobiose stock solution (5.0 mM) were mixed to form a 190  $\mu\text{L}$  prereaction mixture. The reaction was initiated with the addition of 10  $\mu\text{L}$  of NodST stock 1. The concentrations of PAPS and chitobiose were 25  $\mu\text{M}$  and

1 mM, respectively, along with 90 nM NodST in the reaction system. The sample was kept at room temperature overnight, and the enzymatic reaction was allowed to proceed to completion. At this point, PAPS had been completely consumed as evidenced by the ESI-MS spectra, providing a chitobiose-6-OSO<sub>3</sub><sup>-</sup> concentration of 25  $\mu\text{M}$ . A 25  $\mu\text{L}$  aliquot of the reaction solution was quenched in 100  $\mu\text{L}$  of MeOH containing 6.25  $\mu\text{M}$  internal standard. This quenched sample solution was analyzed by selected ion monitoring (SIM) to obtain the single-point normalization factor (31).

**Progress Curve of the Reaction.** The same 190  $\mu\text{L}$  prereaction mixture was generated, and the reaction was initiated with the addition of 10  $\mu\text{L}$  of NodST stock 1. Several 25  $\mu\text{L}$  aliquots were removed and diluted in quenching solution (100  $\mu\text{L}$  of MeOH containing 6.25  $\mu\text{M}$  internal standard) over a 35 min period. The amount of product in each quenched sample solution was quantified by the ESI-MS assay. The reaction percent conversion was determined as a function of time.

**Measurement of  $K_M$  and  $V_{\text{max}}$  for PAPS.** A series of eight prereaction solutions (190  $\mu\text{L}$  each) with eight different PAPS concentrations and a fixed chitobiose concentration were prepared by mixing the PAPS stock solution (12.5 and 125  $\mu\text{M}$ ), the chitobiose stock solution (5.0 mM), and buffer A at eight different ratios. After each reaction was initiated with 10  $\mu\text{L}$  of NodST stock 1, the PAPS concentrations in the eight reaction solutions ranged from 1.25 to 50  $\mu\text{M}$  along with 1 mM chitobiose and 90 nM NodST. After 4 min, a 25  $\mu\text{L}$  aliquot of each reaction solution was quenched in 100  $\mu\text{L}$  of MeOH with 6.25  $\mu\text{M}$   $\Delta\text{D}_i$ -6S. The eight quenched samples were analyzed by ESI-MS, and the amount of product in each sample was quantified. The  $K_M$  and  $V_{\text{max}}$  values were determined by plotting the calculated initial velocity versus PAPS concentration using the GraFit program. The error in these calculations was determined from three replicate experiments.

**Measurement of  $K_M$  and  $V_{\text{max}}$  for Chitobiose.** After the chitobiose stock solution (0.5 and 5.0 mM), PAPS stock solution (125  $\mu\text{M}$ ), and buffer A had been mixed at 10 different ratios, a series of 10 prereaction solutions (190  $\mu\text{L}$  each) with 10 different chitobiose concentrations and a fixed PAPS concentration were prepared. After each reaction had been initiated with 10  $\mu\text{L}$  of NodST stock 1, the final chitobiose concentrations in the 10 reaction solutions ranged from 0.05 to 2.5 mM with 25  $\mu\text{M}$  PAPS and 90 nM NodST. After 4 min, a 25  $\mu\text{L}$  aliquot of each reaction solution was quenched in 100  $\mu\text{L}$  of MeOH with 6.25  $\mu\text{M}$   $\Delta\text{D}_i$ -6S. The 10 quenched samples were analyzed by ESI-MS, and the amount of product in each sample was quantified. The  $K_M$  and  $V_{\text{max}}$  values were obtained by plotting the calculated initial velocity versus chitobiose concentration using the GraFit program. The experiments described above were repeated three times to determine the precision of the assay.

**Product Inhibition Study Using PAP.** A stock solution of NodST (1.3  $\mu\text{M}$ ) (NodST stock 2) was prepared in buffer A. Stock solutions of chitobiose (10 mM), PAPS (12.5–500  $\mu\text{M}$ ), and the product inhibitor PAP (0–30  $\mu\text{M}$ ) were prepared in buffer A. The reaction solution was prepared in a total volume of 200  $\mu\text{L}$ . Four concentrations of PAP were used: 0, 0.75, 1.5, and 3.0  $\mu\text{M}$ . For each of the PAP concentrations, a series of six PAPS concentrations ranging from 1.25 to 50  $\mu\text{M}$  were used, while the chitobiose

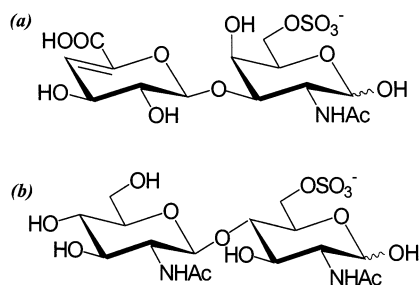


FIGURE 1: (a) Structure of the internal standard  $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-6S ( $\Delta$ D<sub>1</sub>-6S) with  $m/z$  458. (b) Structure of the monitored product chitobiose-6-OSO<sub>3</sub><sup>-</sup> with  $m/z$  503.

concentration was kept constant at 1 mM. Hence, a total of 24 prereaction mixtures (60  $\mu$ L each) were prepared by mixing 20  $\mu$ L of a 10 mM chitobiose stock, a PAPS stock, and a PAP stock of appropriate concentrations. Each reaction was initiated by addition of 140  $\mu$ L of NodST stock 2; thus, the NodST concentration in each reaction was 90 nM. After 4 min, a 25  $\mu$ L aliquot of each reaction solution was quenched in 100  $\mu$ L of MeOH with 6.25  $\mu$ M  $\Delta$ D<sub>1</sub>-6S. The 24 quenched samples were analyzed by ESI-MS, and the amount of product in each sample was quantified. The mode of inhibition was evaluated by analyzing the pattern of the double-reciprocal plots. The  $K_i$  value of PAP versus the substrate PAPS was obtained.

## RESULTS AND DISCUSSION

### *Internal Standard and Single-Point Normalization Factor.*

A critical aspect of the ESI-MS assay is the quantification of the product by introduction of an internal standard in the quenching solution and monitoring of the relative intensity of product ion and internal standard ion by SIM. The intensity ratio of the two ions is related to their concentration ratio through the normalization factor. A single-point normalization factor  $R$  can be determined with eq 1, which can be obtained by analyzing a mixture of the internal standard and the product at a known concentration. The latter is determined by monitoring depletion of a known substrate concentration.

$$R = (I_P/I_{IS})/([product]/[internal\ standard]) \quad (1)$$

where  $I_{IS}$  is the intensity of the internal standard and  $I_P$  is the intensity of the product.

For each sample that was analyzed, the product concentration and the initial reaction velocity  $V_0$  at certain substrate concentrations can be calculated via eqs 2 and 3 using the ESI-MS data ( $I_P/I_{IS}$ ) and the normalization factor  $R$  determined above.

$$[product] = (I_P/I_{IS})[internal\ standard]/R \quad (2)$$

$$V_0 = [product]/T_q \quad (3)$$

where  $T_q$  is the quenching time.

In our NodST assay, a chondroitin disaccharide,  $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-6S ( $\Delta$ D<sub>1</sub>-6S) (Figure 1a), was chosen as an internal standard due to its molecular weight and chemical structure being similar to those of the product, chitobiose-6-OSO<sub>3</sub><sup>-</sup> (Figure 1b). Both compounds are 6-sulfated disaccharides and have similar ionization efficiencies. This

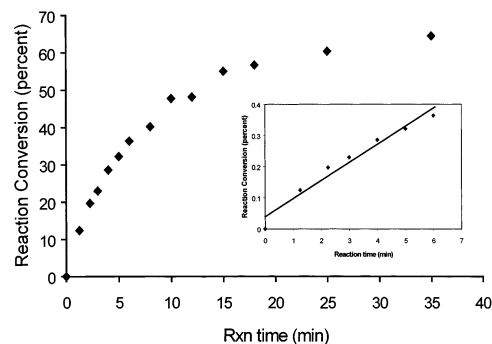


FIGURE 2: Progress curve for determining the optimum reaction time of the NodST enzymatic system ([chitobiose] = 1 mM, [PAPS] = 25  $\mu$ M, [NodST] = 90 nM, and pH 8.0). The inset (from 0 to 6 min) is shown as the expanded region.

ensures a linear relationship of their concentrations and ion intensity ratios. The two ions that were monitored were [ $\Delta$ D<sub>1</sub>-6S-H]<sup>-</sup> and [chitobiose-6-OSO<sub>3</sub><sup>-</sup>-H]<sup>-</sup>, at  $m/z$  458 and 503, respectively. Since the product chitobiose-6-OSO<sub>3</sub><sup>-</sup> is not commercially available, a known product concentration was generated by allowing the enzymatic reaction to run to completion. ESI-MS was used to ensure the completion of the reaction by monitoring the disappearance of the substrate peak, [PAPS-H]<sup>-</sup>, at  $m/z$  506. The chitobiose-6-OSO<sub>3</sub><sup>-</sup> concentration is then equivalent to the initial PAPS concentration. This allowed for the determination of the average single-point normalization factor for the NodST reaction. This was determined to be 2.7 and only varied slightly ( $\pm 0.1$ ) during the course of the study.

**Reaction Progress Curve.** Before the kinetic parameters for PAPS and chitobiose in the NodST enzymatic system were measured, reaction progress curve experiments were performed to determine the optimum enzyme concentration and reaction quenching time for the kinetic study. A progress curve was obtained by monitoring the amount of product versus time using the ESI-MS assay (Figure 2). The linear region of 0–6 min was obtained, and the optimum reaction time was determined to be 4 min with a substrate conversion of approximately 27% and a NodST concentration of 90 nM at pH 8.0. Since the quenching time resided in the linear region, the initial velocity was calculated by dividing the product concentration by the reaction time.

**Measurement of  $K_M$  and  $V_{max}$  for PAPS.** On the basis of the  $K_M$  value of 4.3  $\mu$ M previously determined by a TLC assay (16), a PAPS concentration range of 1.25–50  $\mu$ M (0.25–5.0 $K_M$ ) was used in this study. For measurement of  $K_M$  and  $V_{max}$  for PAPS, the concentration of this substrate was varied in each reaction while the other substrate, chitobiose, was kept at a fixed saturating concentration. The concentration of chitobiose-6-OSO<sub>3</sub><sup>-</sup> was quantified using the previously determined normalization factor and the ESI-MS data. The initial reaction velocity was calculated and plotted versus the PAPS concentration to obtain the corresponding  $K_M$  and  $V_{max}$  values, via nonlinear regression analysis. Figure 3a shows the saturation plot of initial velocity versus PAPS concentration obtained from the ESI-MS data, which is for one experiment from a total of three replicate experiments (see the Supporting Information). The initial velocity reached a maximum at a PAPS concentration of approximately 60–100  $\mu$ M, and the average  $K_M$  and  $V_{max}$  values for PAPS were determined to be  $6.7 \pm 0.6 \mu$ M and

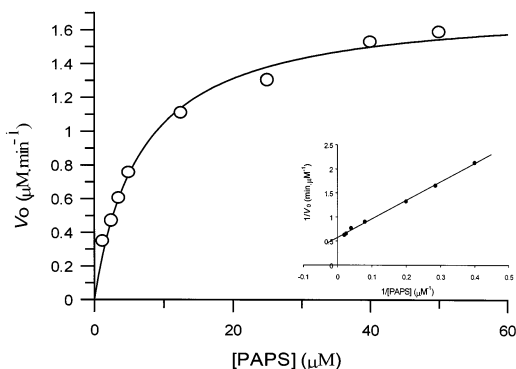


FIGURE 3: Saturation plot of  $V_0$  vs [PAPS]. The inset is a double-reciprocal plot of  $1/V_0$  vs  $1/[PAPS]$  ([PAPS] = 1.25, 2.5, 3.5, 5, 12.5, 25, 40, and 50  $\mu\text{M}$ , [chitobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

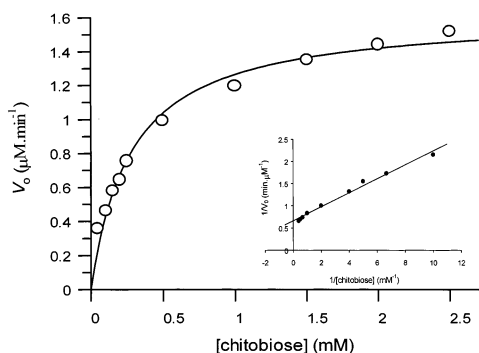


FIGURE 4: Saturation plot of  $V_0$  vs [chitobiose]. The inset is a double-reciprocal plot of  $1/V_0$  vs  $1/[chitobiose]$  ([chitobiose] = 0.05, 0.1, 0.15, 0.2, 0.25, 0.5, 1, 1.5, 2, and 2.5 mM, [PAPS] = 25  $\mu\text{M}$ , [NodST] = 90 nM, and pH 8.0).

$1.75 \pm 0.05 \mu\text{M min}^{-1}$ , respectively. The  $K_M$  value was reasonably consistent with that determined using the TLC assay (4.3  $\mu\text{M}$ ) (16), validating the ESI-MS assay as a reliable and accurate method for determining enzyme kinetic parameters for NodST. The corresponding double-reciprocal plot (Figure 3b), based on the double-reciprocal equation, had an  $R^2$  value of 0.998, which further verified the precision of the methodology. Hence, this method can be used to obtain the  $K_M$  and  $V_{\max}$  values for the other substrate, chitobiose.

**Measurement of  $K_M$  and  $V_{\max}$  for Chitobiose.** Several preliminary experiments with a limited number of chitobiose concentrations were performed to estimate the  $K_M$  value and determine the proper chitobiose concentration range for the study. On the basis of preliminary assays estimating a  $K_M$  value of 0.25 mM, a chitobiose concentration range of 0.05–2.5 mM ( $0.2$ – $10.0K_M$ ) was used. For the measurements of  $K_M$  and  $V_{\max}$  for chitobiose, PAPS was kept at a fixed, saturating concentration of 25  $\mu\text{M}$ , while the concentration of chitobiose was varied from 0.05 to 2.5 mM. The product, chitobiose-6-OSO<sub>3</sub><sup>-</sup>, was quantified using the normalization factor and ESI-MS data, and the initial reaction velocity was calculated and plotted versus the chitobiose concentration (Figure 4a). In this case, the initial reaction velocity approached a maximum at a chitobiose concentration between 2.5 and 5.0 mM. A nonlinear regression analysis of the saturation plot of initial velocity versus chitobiose concentration was performed, yielding an average  $V_{\max}$  value of  $1.62 \pm 0.05 \text{ mM min}^{-1}$  and a  $K_M$  value of  $0.28 \pm 0.03 \text{ mM}$ . The results, which were generated from three replicate experiments (see the Supporting Information), agreed well

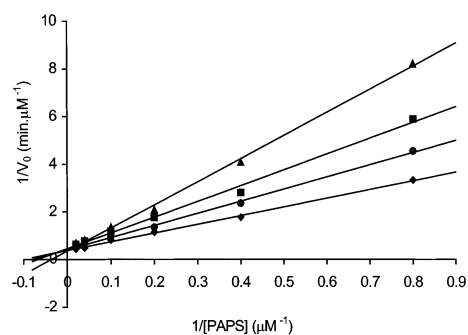


FIGURE 5: Double-reciprocal plot of  $1/V_0$  vs  $1/[PAPS]$  at different PAP concentrations: 0  $\mu\text{M}$  PAP ( $\blacktriangle$ ), 0.75  $\mu\text{M}$  PAP ( $\blacksquare$ ), 1.5  $\mu\text{M}$  PAP ( $\bullet$ ), and 3.0  $\mu\text{M}$  PAP ( $\blacklozenge$ ) ([PAPS] = 1.25, 2.5, 5, 10, 25, and 50  $\mu\text{M}$ , [chitobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

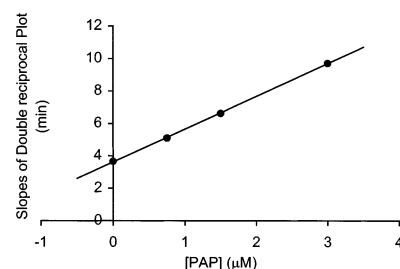


FIGURE 6: Secondary plot of the slope of each line in the double-reciprocal plot vs [PAP].

with previously published results for chitobiose in the NodST enzymatic reaction (22). The high precision of the results is underscored by the good linearity of the corresponding double-reciprocal plot (Figure 4b), which gave an  $R^2$  value of 0.988.

**Product Inhibition Study Using PAP.** NodST has been used as a model enzyme for the mammalian GlcNAc-6-*O*-sulfotransferases. Inhibitor studies with NodST are expected to provide critical information for the design of sulfotransferase inhibitors with potential anti-inflammatory and anti-cancer activities. We endeavored to demonstrate that our assay is amenable to inhibition studies and simultaneously gain insight into NodST's mechanism of action. One of the products of the bisubstrate sulfotransferase reaction, PAP, was previously reported to exhibit competitive inhibition of NodST with respect to an inhibition constant,  $K_i$ , of 1.36  $\mu\text{M}$  (16).

To validate the ESI-MS assay, we studied NodST inhibition using PAP by evaluating the double-reciprocal plots generated using different inhibitor concentrations. We varied the concentrations of one substrate PAPS (from 1.25 to 50  $\mu\text{M}$ ) and the inhibitor PAP (from 0 to 3.0  $\mu\text{M}$ ), while the concentration of the other substrate, chitobiose, was kept constant at 1 mM. The same assay method was applied to quantify the product and calculate the initial velocity. Figure 5 shows the double-reciprocal plots obtained from the ESI-MS data. The four lines representing four different PAP concentrations shared a common  $Y$ -intercept, unambiguously indicating a competitive inhibition pattern for PAP.

Figure 6 shows the secondary plot for PAP in our inhibition study, which gave a  $K_i$  value of 1.80  $\mu\text{M}$  with respect to the substrate PAPS at a saturating chitobiose concentration. The result was consistent with that determined previously using the TLC assay (16). This efficient mass

spectrometric method is currently being used to determine the inhibition mode and kinetic parameters for other synthetic inhibitors of NodST (16).

## CONCLUSIONS

A novel ESI-MS kinetics assay has been applied to study NodST, a model for the family of mammalian carbohydrate sulfotransferases of therapeutic interest. The ESI-MS method is especially useful in characterizing enzymes for which no spectrophotometric assay is feasible. With the availability of a proper internal standard that is similar to one of the reaction components in both structure and ionization efficiency, we can utilize the single-point normalization factor to simplify and streamline the ESI-MS analysis. The kinetic parameters for the NodST obtained from this ESI-MS assay were in excellent agreement with previously reported results. Since radiolabeled materials are not required in the ESI-MS assay, and because the analysis time is comparable to standard UV techniques, the mass spectrometry-based assay is expected to be competitive with, and in some cases more convenient than, traditional methods. This technique is also an efficient way for determining the inhibition mode of NodST inhibitors and performing mechanistic studies. Future efforts will be focused on determining  $K_i$  values of a synthetic library of possible inhibitors and application of the methodology to further investigation of therapeutically interesting enzymes.

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## SUPPORTING INFORMATION AVAILABLE

Replicate experimental data for the  $K_M$  and  $V_{max}$  measurement of both substrates, including the donor substrate PAPS and the acceptor substrate chitobiose. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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