Kinetic measurements and mechanism determination of Stf0 sulfotransferase using mass spectrometry

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Abstract

Mycobacterial carbohydrate sulfotransferase Stf0 catalyzes the sulfuryl group transfer from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to trehalose. The sulfation of trehalose is required for the biosynthesis of sulfolipid-1, the most abundant sulfated metabolite found in Mycobacterium tuberculosis. In this paper, an efficient enzyme kinetics assay for Stf0 using electrospray ionization (ESI) mass spectrometry is presented. The kinetic constants of Stf0 were measured, and the catalytic mechanism of the sulfuryl group transfer reaction was investigated in initial rate kinetics and product inhibition experiments. In addition, Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry was employed to detect the noncovalent complexes, the Stf0–PAPS and Stf0–trehalose binary complexes, and a Stf0–3'-phosphoadenosine 5'-phosphate–trehalose ternary complex. The results from our study strongly suggest a rapid equilibrium random sequential Bi-Bi mechanism for Stf0 with formation of a ternary complex intermediate. In this mechanism, PAPS and trehalose bind and their products are released in random fashion. To our knowledge, this is the first detailed mechanistic data reported for Stf0, which further demonstrates the power of mass spectrometry in elucidating the reaction pathway and catalytic mechanism of promising enzymatic systems.

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Sulfation of biomolecules is a posttranslational modification that is widely observed from bacteria to mammals and regulates a variety of cellular communication events [1,2]. Sulfotransferases (STs)1 are enzymes that are responsible for transferring the sulfuryl group from a universal sulfuryl group donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to numerous acceptor substrates including proteins [3,4], carbohydrates [1,5], and low-molecular-weight metabolites [6,7]. They also play significant roles in modulating normal and patho-

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1 Abbreviations used: ST, sulfotransferase; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PAP, 3'-phosphoadenosine 5'-phosphate; 3'PB motif, 3'-phosphate binding motif; EST, estrogen sulfotransferase; ER, estrogen receptor; 5'PSB-loop, 5'-phosphosulfate binding loop; HNDST-1, heparan sulfate N-deacetylase/N-sulfotransferase 1; HS3OST-1, heparan sulfate 3-O-sulfotransferase isoform 1; PST, phenol sulfotransferase; FST, flavonol sulfotransferase; SL-1, sulfolipid-1; T2S, trehalose-2-sulfate; ESI-MS, electrospray ionization mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; FT-ICR MS, Fourier transform ion cyclotron resonance mass spectrometry; GST, glutathione S-transferase; PGI, phosphoglucomutase; PGM, phosphoglucomizerase; SIM, selected ion monitoring; GalNAc, N-acetylgalactosamine; ΔDi-6S, α-AUA-[1 → 3]-GalNAc-6S.
genic biological processes. For example, bacterial sulfotransferase NodH is involved in the biosynthesis of sulfated nod factor which induces root nodulation in the symbiotic host plant [8], while the interaction between L-selectin and its sulfated glycoprotein ligands contributes to the leukocyte immigration into inflamed tissues and results in acute and chronic inflammation [9,10]. The sulfated chemokine receptor CCR5, modified by tyrosine protein sulfotransferases, binds to HIV gp120 during the HIV infection process [11]. Similarly, the sulfation of estradiol, catalyzed by estrogen sulfotransferase (EST), can activate the estrogen receptor (ER) and is believed to underlie the genesis of breast cancer [12,13]. The discovery that sulfotransferases participate in various disease states has positioned the enzymes as novel therapeutic targets [1,2].

The mechanistic information for sulfuryl group transfer is critical in understanding the functions of STs in vivo. Studies of the structure and catalytic mechanism of this enzyme class have been most extensively focused on eukaryotes, particularly on cytosolic STs. Crystallographic and mutational analyses have provided critical information about the active-site residues involved in ST catalysis [14–22]. For example, the crystal structures of EST have been solved in the presence of 3'0-phosphoadenosine 5'0-phosphate (PAP) and estradiol [14], PAP and vanadate [15], and mostly recently with the donor substrate PAPS [16]. These EST structures, along with site-directed mutagenesis work, have unraveled the central catalytic roles for Lys and Thr in the 5'0-phosphosulfate binding loop (5'0PSB-loop) and for Ser in the 3'0-phosphate binding motif (3'PB motif). Structural studies of the ST domain of the heparan sulfate N-deacetylase/N-sulfotransferase 1 (HNDST-1) [21] and the heparan sulfate 3'-O-sulfotransferase isofrom 1 (HS3OST-1) [22] associated with PAP further verified Lys, His, and Ser as highly conserved residues. The relative orientation of the bound molecules in the solved ST structures indicated a characteristic transition state for a S_N2-like in-line displacement reaction [16,22,23]. In addition, direct kinetic analyses have been performed to investigate the catalytic mechanism of cytosolic STs. Varin and coworkers [24,25] determined that phenol sulfotransferases (PSTs) and flavonol sulfotransferases (FSTs) followed an ordered Bi-Bi mechanism using initial rate kinetics and product inhibition. Recently, a random Bi-Bi mechanism was elucidated for EST [26] and an insect sulfotransferase, retinol dehydratase [27]. The kinetic studies point toward a sequential mechanism with ternary complex formation for cytosolic STs, and this finding is consistent with a previously proposed in-line transfer mechanism based on the structural features of cytosolic STs.

In comparison, only sparse information on the structure and mechanism of bacterial STs has been obtained. Only one bacterial ST, NodH, from *Rhizobium meliloti*, has been assigned a hybrid random ping-pong mechanism using kinetic analysis and characterization of the sulfated enzyme intermediate [28]. In this mechanism, both donor and acceptor substrates bind independently and randomly at two different sites on NodH. A flexible domain which presumably contains the 5'PSB-loop of NodH is sulfated by PAPS bound in the first site, yielding the substituted enzyme intermediate.

Recently, a novel mycobacterial sulfotransferase, termed Stf0, was identified and characterized by Mougous et al. [29]. This enzyme initiates the biosynthesis of sulfolipid-1 (SL-1), the most abundant sulfated metabolite and potential virulence factor found in *Mycobacterium tuberculosis* [30–33]. Stf0 was demonstrated to catalyze the sulfuryl group transfer from PAPS to trehalose to form trehalose-2-sulfate (T2S), the core disaccharide of SL-1 (Scheme 1). The crystal structure of Stf0 was solved in the presence of trehalose,
which revealed the molecular basis of trehalose recognition. As the first prokaryotic ST to be structurally characterized and the first carbohydrate ST that has its structure solved in the presence of an acceptor substrate, Stf0 is an interesting target for catalytic mechanism determination.

Soft ionization methods such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization mass spectrometry have been demonstrated to be techniques complementary to conventional spectroscopic methods for enzyme kinetics studies [34–42]. A facile and broadly applicable ESI-MS assay developed by Leary and co-workers has been implemented for kinetic analyses and substrate specificity evaluation of several enzyme systems such as glutathione S-transferase [36], hexokinase [37], PGI and PGM [38,39], and NodH sulfitotransferase [28,40,41]. This assay does not require chromophoric reaction components and the total analysis time is comparable to that of traditional methods. Additionally, assays for analyzing multiple substrates [41] and multiple enzyme tandem reactions were developed using the ESI-MS technique and the latter were successfully applied to monitor the biosynthetic pathway of the antibiotic novobiocin [42].

Herein we report the complete kinetic characterization of the Stf0-catalyzed sulfuryl group transfer reaction from PAPS to trehalose. The product, trehalose-2-sulfate, was quantified in relation to an internal standard using single-point normalization factors. The enzyme kinetic parameters for Stf0 were rapidly measured using the ESI-MS assay. The catalytic mechanism of Stf0 was determined by initial rate kinetics and the latter were successfully applied to monitor the catalytic reaction using FT-ICR mass spectrometry. These are the first data reported on the catalytic mechanism of mycobacterial carbohydrate sulfitotransferase Stf0 and the data are highly suggestive of a rapid equilibrium random sequential Bi-Bi mechanism. Our research on the enzymatic properties of Stf0 provides a platform for understanding the roles of STs in bacteria. Considering that prokaryotic carbohydrate STs (e.g., NodH and Stf0) are functionally analogous to eukaryotic carbohydrate STs that have a central role in numerous disease states, insights gained from the structure and mechanism study of Stf0 will greatly facilitate ST-targeted drug design [43].

Materials and methods

General materials and methods

Trehalose, 3′-phosphoadenosine 5′-phosphate, and the internal standard α-AUA-[1 → 3]-GalNAc-6S (ΔD,-6S) were purchased from Sigma (St. Louis, MO) and used without further purification, while 3′-phosphoadenosine-5′-phosphosulfate was purchased from Calbiochem (San Diego, CA). Stf0 was overexpressed and purified as previously described [29]. Coomassie plus protein assay reagent (950 mL) and albumin standard (10 × 1 mL) were purchased from Pierce (Rockford, IL). All enzymatic reactions and mass spectrometric analyses were carried out at ambient temperature.

PAPS purification

Given that commercial PAPS was found to contain 15% PAP, a product inhibitor of the Stf0 enzyme, further purification of PAPS was performed to ensure accurate results for Stf0 kinetics. PAPS was purified via a 1-mL HiTrap Q HP anion-exchange column (Amersham, Piscataway, NJ) using ddH2O and 2 M NH4OAc, pH 6.8, in a mixture gradient of 2–100% 2 M NH4OAc over 90 min at constant 4 °C. A BioLogic LP system (Bio-Rad, Hercules, CA) was used to perform the chromatography. Aliquots of 700 μL were automatically collected every 90 s. The presence of PAPS and PAP in the eluate was monitored using UV absorption at 254 nm. Fractions containing separated PAPS were collected and subsequently dialyzed in ddH2O using 3-μL-capacity dialysis tubes with 100 MW cutoff (Spectrum Lab, Rancho Dominguez, CA) at 4 °C. The total concentration of PAPS after desalination was determined by UV at 260 nm (ε = 15,100 M−1 cm−1). The purity of PAPS after dialysis was measured to be 90% using mass spectrometry as previously described [44].

Instrumentation

 Ion trap mass spectrometry

A LCQ classic ion trap mass spectrometer equipped with an ESI source and a HPLC pump (Thermo-Finnigan, San Jose, CA) was used for kinetic analyses. Approximately 10 μL of each sample solution was injected into a 5-μL injection loop and delivered via an LC pump using a mixture of water and methanol at a flow rate of 20 μL min−1. The capillary temperature and the spray voltage were kept at 200 °C and 3.2 kV, respectively. The ions of interest were monitored in the negative ion mode and signal intensity was optimized using the automatic tuning option on the instrument. When the signal intensity for one sample decreased from approximately 5 × 105 detector counts per scan to 5 × 103 detector counts per scan, indicating the consumption of the sample, the next sample was injected. Selected ion monitoring (SIM) mode was employed for quantification and the peak area for each SIM ion was automatically integrated using the Qual Browser program. The integrated peak area of the product ion and the internal standard ion were used to determine their peak area ratio (A_P/A_IS).
FT-ICR mass spectrometry

A Bruker FT-ICR mass spectrometer equipped with an actively shielded 7-Tesla superconducting magnet was used for analyzing the Stf0-ligand noncovalent complexes. Solutions were infused into an Apollo electrospray source at a rate of 2 μL min⁻¹. The N₂ nebulizing and drying gas pressures were maintained at 50 and 25 psi, respectively. The bias on the glass capillary was kept at 4600 V and a 182 °C drying gas was used to assist the desolvation process. Further desolvation was achieved by collisions of the ions with neutral buffer gas at the nozzle–skimmer region using a ~150-V capillary exit accelerating voltage. A throttle valve was installed at the nozzle–skimmer region and the pressure was adjusted to 1 × 10⁻⁵ mbar. Ions were externally accumulated in a radiofrequency-only hexapole for 1 s and four ion packages were transferred into the ICR cell. Excessive kinetic energy was removed by colliding the ions with Ar pulsed into the cell to a pressure of ~10⁻⁵ mbar and the trapping voltage was decreased to ~0.5 V. A series of pump downs were applied to lower the pressure in the cell to ~10⁻¹⁰ mbar for ion detection, which was achieved by chirp excitation and broad-band data acquisition using an average of 16 time domain transients containing 8 k data points. The original time domain free induction decay spectra were zero-filled, Gaussian-multiplied, and Fourier-transformed. The parameters of the ESI source, ion optics, and cell were tuned for the best signal to noise ratio and kept for the same set of experiments. All the data were acquired and processed using Bruker Xmass v6.0.0 software (Billerica, MA).

Enzyme kinetics

Quantification method

Single-point normalization factors (R factors) [37,40] were used for product quantification. As described in Eq. (1), the peak area integration ratio of the product ion and the internal standard ion (AP/AS) is related to their concentration ratio through an R factor.

\[ R = \frac{AP}{AS} \times \frac{[\text{Product}]}{[\text{Internal Standard}]} \]  

(1)

In our kinetic study, a chondroitin disaccharide, α-DUA-[1 → 3]-GalNAc-6S (ΔD₆₅₆₆S), was used as an internal standard because of its similar molecular weight and chemical structure relative to the product, T2S. The two ions monitored were [ΔD₆₆₆₆S⁻⁻] and [T2S⁺⁺], at m/z 421 and m/z 503, respectively.

To obtain R factors at different trehalose concentrations, nine samples containing nine trehalose concentrations (0, 2, 4, 6, 8, 10, 15, 20, 30 mM) and a known product and internal standard concentration (50 μM) were prepared. SIM was performed in triplicate on each sample to obtain AP/AS, and R factors at different trehalose concentrations were calculated using Eq. (1).

For each quenched reaction sample analyzed, the product concentration can be calculated via Eq. (2) using the ESI-MS data (AP/AS) and the corresponding R factor determined at the trehalose concentration used for the reaction.

\[ [\text{Product}] = \frac{(AP/AS)}{R} \times \frac{[\text{Internal Standard}]}{IS} \]  

(2)

Kinetic constant measurements

All reactions were assayed in 30-μL 10 mM NH₄OAc buffer, pH 7.5. Reactions were initiated by the addition of Stf0 and terminated at specified time points by quenching 10-μL aliquots in 40 μL of methanol containing 12.5 μM ΔD₆₆₆₆S at 4 °C. The quenched samples were subsequently analyzed by ESI-MS. For the determination of kinetic parameters for trehalose, Stf0 and PAPS were maintained at 112 nM and 1 mM, respectively, and trehalose was varied over a concentration range of 2–30 mM. For the determination of kinetic parameters for PAPS, the concentrations used for Stf0 and trehalose were 112 nM and 30 mM, respectively, and PAPS concentration was varied from 10 to 500 μM. Both saturation and double-reciprocal plots were generated via nonlinear fit to Michaelis–Menten equation using the GraFit program (Version 4.0.12; Erithacus Software, Horley, Surrey, UK). The Kₘ and kₗₘ values for trehalose and PAPS were subsequently determined using the same program. Each experiment was carried out in triplicate.

Initial rate kinetics analysis

The mechanism of Stf0 was determined using trehalose and PAPS as substrates. Stf0 was added to a final concentration of 250 nM. The concentration of PAPS was varied from 10 to 50 μM, and the concentration of trehalose ranged from 2 to 10 mM. Partial substrate inhibition by trehalose and PAPS is negligible at this concentration range. Initial velocities were determined under each of the 25 conditions defined by a 5 × 5 matrix of substrate concentrations. The catalytic mechanism of Stf0 was evaluated by plotting 1/V₀ as a function of both 1/[PAPS] at different fixed trehalose concentrations and 1/[trehalose] at different fixed PAPS concentrations using the GraFit program. Experiments were carried out in triplicate.

Product inhibition study

For the product inhibition study of PAP versus PAPS, Stf0 was added to a final concentration of 100 nM, while trehalose was kept at a constant concentration of 10 mM. The concentration of PAPS was varied from 10 to 50 μM, and the five different concentrations used for PAP were 0, 10, 20, 50, and 100 μM. For the product inhibition study of PAP versus trehalose, the concentrations used for Stf0 and PAPS were 100 nM and 200 μM, respectively. The concentration
of trehalose was varied from 2 to 10 mM, and the five PAP concentrations used were 0, 50, 100, 150, and 200 μM. In each case, the 25 quenched samples were analyzed by ESI-MS to obtain initial velocities. The mode of inhibition and the $K_i$ value were determined by fitting data into the GraFit program and displayed as a double-reciprocal plot. Each experiment was carried out in triplicate.

Detection of the Stf0 noncovalent complexes

A series of five solutions were prepared in 50 mM NH$_4$OAc, pH 7.5. Solution 1 contained 10 μM Stf0; solution 2 contained 100 μM trehalose and 10 μM Stf0; solution 3 contained 20 μM PAP and 10 μM Stf0; solution 4 contained 20 μM PAPS and 10 μM Stf0; solution 5 contained 100 μM trehalose, 20 μM PAP, and 10 μM Stf0. Each solution was previously incubated on ice for 20 min and then infused into the ESI-FT-ICR mass spectrometer using positive ion mode detection. The spray chamber was wrapped with ice bags to prevent the protein sample from precipitating out from the buffer solution.

Results and discussion

Product quantification

The trehalose concentrations used in the Stf0 kinetic analysis were in the range of 2–30 mM which was much higher than the concentrations of the other reaction components (PAPS, PAP, and T2S), including the internal standard. Trehalose in this concentration range will affect the relative ionization intensity ratio of the product and the internal standard, therefore affecting the $R$ factor. To achieve accurate product quantification, $R$ factors were measured at different trehalose concentrations and subsequently used in the kinetic analysis.

As illustrated in Fig. 1, the average $R$ factor was determined to be 0.6 when no trehalose was added to the reaction solution, and it increased gradually over the trehalose concentration range of 0–15 mM. The highest $R$ factor was observed at a trehalose concentration of 15 mM. Over the trehalose concentration range of 15 to 30 mM, the $R$ factor was in a relatively steady state. This is likely due to a higher ionization suppression by trehalose on the internal standard ion than on the product ion, resulting in the increased $R$ factor with increased trehalose concentration.

Kinetic constant measurements

The kinetic parameters of Stf0 were defined with either PAPS or trehalose as the variable substrate (Figs. 2A and B). The results were obtained by fitting data into the Michaelis-Menten equation. No enzyme inhibition was observed over the concentration ranges of PAPS and trehalose used. Typical hyperbolic saturation was observed when PAPS was the variable substrate.

![Fig. 1. Product quantification was carried out using different normalization factors ($R$ factors) at different trehalose concentrations. Shown here is the plot of $R$ factor versus [trehalose].](image1)

![Fig. 2. Measurements of kinetic constants for Stf0-catalyzed sulfuryl group transfer reaction from PAPS to trehalose. (A) Saturation plot of $V_0$ versus [PAPS] and double-reciprocal plot of $1/V_0$ versus $1/[\text{PAPS}]$ (inset) at a fixed trehalose concentration (30 mM). (B) Saturation plot of $V_0$ versus [trehalose] and double-reciprocal plot of $1/V_0$ versus $1/[\text{trehalose}]$ (inset) at a fixed PAPS concentration (1 mM).](image2)
(trehalose concentration was held constant at 30 mM), resulting in an apparent $K_m$ of $18 \pm 3 \mu M$ for PAPS. Under these conditions, a maximum rate constant of $96 \pm 2 \text{ min}^{-1}$ was observed and this is in good agreement with the rate constant measured in parallel experiments carried out using 30 mM trehalose under saturating PAPS conditions ($98 \pm 2 \text{ min}^{-1}$). When trehalose was the variable substrate and PAPS concentration was held constant at 1 mM, a Michaelis–Menten curve fit of the data up to 30 mM trehalose provided an apparent $K_m$ of $15 \pm 1 \text{ mM}$ and a $k_{cat}$ of $134 \pm 10 \text{ min}^{-1}$ for trehalose. The kinetic constants for trehalose are in excellent agreement with those obtained by the TLC assay used previously [29]. The results for PAPS are the first reported values to date and the $K_m$ value of PAPS is similar to those measured for other STs [24–27,40]. The $K_m$ values of trehalose and PAPS indicate that the donor substrate PAPS has a much higher binding affinity to Stf0 than the acceptor substrate trehalose. This is reasonable given that trehalose is present at high physiological concentrations in vivo [45], suggesting that the enzyme is optimized to function at physiological levels of trehalose.

**Initial rate kinetics analysis**

To examine whether a sequential or a ping-pong Bi-Bi mechanism is operative in Stf0, a classical initial velocity study of the sulfuryl group transfer reaction was performed. Initial reaction rates were obtained using data at low reaction conversion to avoid complicated reaction conditions such as substrate depletion, product inhibition, and reverse reaction. Preliminary experiments were performed to ensure that inhibition of both substrates was negligible within the chosen concentration ranges. The initial rate of the reaction was determined as a function of both substrates (Fig. 3) and as a function of trehalose at different fixed concentrations of PAPS (data not shown). The kinetic data were fitted into the rate equation of two mechanistic models (sequential and ping-pong mechanisms) of bisubstrate reactions using the GraFit program, and the best fit was obtained in the case of a sequential mechanism model. Fig. 3 shows the double-reciprocal plot of $1/V_0$ versus $1/[PAPS]$ and the five lines representing different trehalose concentrations intersect at one point. In the sequential mechanism, Stf0 binds to trehalose and PAPS to form a ternary complex, catalyzes the sulfuryl group transfer between the two substrates inside the complex, and subsequently releases the reaction products T2S and PAP.

**Product inhibition study using PAP**

We tested the inhibition effect of PAP on the sulfotransferase activity of Stf0 to obtain more information about the catalytic mechanism. The inhibition results were obtained by fitting data into the rate equation of three inhibition patterns (competitive, noncompetitive, and mixed-type inhibitions) using the GraFit program. PAP was found to be a good inhibitor to the donor substrate PAPS when trehalose concentration was kept constant at 10 mM. The best data fit was obtained in the case of a competitive inhibition model. The double-reciprocal plot of $1/V_0$ versus $1/[PAPS]$ at different fixed trehalose concentrations ([trehalose] = 2 mM, [trehalose] = 4 mM, [trehalose] = 6 mM, [trehalose] = 8 mM, and [trehalose] = 10 mM).
The apparent inhibition constant, $K_i$, was determined to be $405 \pm 26 \text{ M}$. These data taken together support a sequential Bi-Bi mechanism for Stf0. There are two distinctive binding sites on Stf0 for PAPS and trehalose; PAP and PAPS share and compete for one of the binding sites, while T2S and trehalose share and compete for the other binding site.

Detection of Stf0-associated noncovalent complexes

An important feature of a sequential Bi-Bi mechanism is the formation of a ternary complex, so-called because it contains the enzyme and both substrates in a single complex. Detection of the ternary complex intermediate of a transferase enzyme and its substrates is highly supportive of a sequential catalytic mechanism adopted by the enzyme [46]. Unfortunately, most group transfer reactions have a relatively high reaction turnover number, and the enzyme–substrates ternary complex is rapidly converted to the free enzyme and the reaction products. Therefore, the ternary complex may not exist in the reaction solution long enough to be detected. This explanation is probably the reason that the Stf0–PAPS–trehalose complex could not be detected when Stf0 was incubated with PAPS and trehalose. However, a ternary complex between Stf0, PAP, and trehalose will accumulate in solution given that Stf0 follows a sequential Bi-Bi mechanism and PAP binds to the PAPS binding site on Stf0. This Stf0–PAP–trehalose complex is analogous to the Stf0–PAPS–trehalose complex formed in the authentic reaction.

To verify the sequential mechanism of Stf0 catalysis, the enzyme was incubated with both PAP and trehalose in NH4OAc buffer. For comparison, Stf0 was also incubated with PAP, PAPS, and trehalose, individually. As a control, Stf0 (10 μM) was first sprayed from NH4OAc buffer. As shown in Fig. 5A, several charge states (10+, 11+, and 12+) were observed. No dimer or higher multimeric states were observed in the mass spectrum, which suggests that Stf0 exists as a monomer in solution. When 10 μM Stf0 was incubated with 20 μM PAP, a mass shift was observed at $m/z$ 2938 in the mass spectrum (Fig. 5B). The mass difference between [Stf0 + PAP]11+ and [Stf0]11+ was calculated to be 427 Da, which corresponds to the noncovalent adduction PAP. The mass spectra of 10 μM Stf0 incubated with 20 μM PAPS (Fig. 5C) also shifted to $m/z$ 2945, a mass difference of 507 Da indicating the formation of Stf0–PAPS binary complex. Fig. 5D represents the mass spectrum of 10 μM Stf0 incubated with 100 μM trehalose. A fivefold excess of trehalose was used because of its relatively low binding affinity to Stf0 in solution. Compared to the spectrum of Stf0, an additional ion was observed at $m/z$ 2930, which was determined to be [Stf0 + trehalose]11+ based on the mass difference of 341 between it and [Stf0]11+. This also clearly indicates the formation of Stf0–trehalose noncovalent complex. Finally, 10 μM Stf0 was incubated with 20 μM PAP and 100 μM trehalose to identify the Stf0–PAP–trehalose ternary complex (Fig. 5E). In addition to the two binary complex ions, [Stf0 + PAP]11+ at $m/z$ 2938 and

![Fig. 4. Product inhibition study of Stf0 by PAP. (A) Double-reciprocal plots of $1/V_0$ versus $1/[PAPS]$ at different fixed PAP concentrations. Trehalose concentration was fixed at 10 mM. (O [PAP] = 0 μM, ● [PAP] = 10 μM, □ [PAP] = 20 μM, ■ [PAP] = 50 μM, and △ [PAP] = 100 μM). (B) Double-reciprocal plots of $1/V_0$ versus $1/[trehalose]$ at different fixed PAPS concentrations. PAPS concentrations was fixed at 200 μM. (O [PAP] = 0 μM, ● [PAP] = 20 μM, □ [PAP] = 50 μM, ■ [PAP] = 100 μM, and △ [PAP] = 200 μM).](image)
[Stf0 + trehalose]$^{11+}$ at $m/z$ 2930, another ion was observed at $m/z$ 2969. The mass difference between this ion and the [Stf0]$^{11+}$ was calculated to be 768 Da, which corresponds to the total molecular weight of PAP and trehalose. Thus, the noncovalent cluster was clearly addressed to be [Stf0 + PAP + trehalose]$^{11+}$. This result strongly supports the formation of Stf0–PAP–trehalose ternary complex and hence the sequential Bi-Bi catalytic mechanism of Stf0. As depicted in the spectra, there was no charge redistribution for Stf0 upon binding to these substrates or product, which suggests that the ligands may bind to Stf0 as the neutral forms [44]. As demonstrated in Figs. 5C and D, PAPS was able to bind to Stf0 without the existence of trehalose and vice versa. These data are more supportive of a random binding mode of substrates to the enzyme instead of a compulsory-order binding mode, pointing to a random sequential Bi-Bi mechanism of Stf0.

**Mechanism of Stf0 catalysis**

Sequential Bi-Bi and ping-pong Bi-Bi are the most commonly occurring catalytic mechanisms for sulfotransferase reactions. In a sequential Bi-Bi mechanism, both substrates bind to the enzyme to form a ternary complex before sulfuryl group transfer and product release occurs. Sequential mechanisms can be further classified as ordered sequential or random sequential mechanisms depending on whether there is a compulsory order for the binding of the donor and acceptor substrates and the release of their products. A ping-pong mechanism involves a covalently substituted enzyme intermediate, in which the sulfuryl group is transferred in a double-displacement pathway and a product is released between substrate additions.

Our data in the initial rate kinetics study provide strong evidence for a sequential Bi-Bi catalytic mechanism for Stf0. The product inhibition patterns of PAP obtained are also consistent with a ternary complex mechanism. However, neither the data generated from the initial velocity analysis nor that from the inhibition study of Stf0 by PAP could differentiate between an ordered and a random mechanism. At this point, the two possible sequential mechanisms for Stf0 could be a rapid equilibrium random sequential Bi-Bi mechanism or a steady state ordered sequential Bi-Bi mechanism. A rapid equilibrium ordered sequential Bi-Bi mechanism is ruled out by the fact that the lines in the $1/V_0$ versus
1/[PAPS] plots (Fig. 3) and the 1/V₀ versus 1/[trehalose] plots (data not shown) do not intersect on the 1/V₀ axis [26,46]. The identification of Stf0-associated noncovalent complexes further substantiates the sequential Bi-Bi mechanism determined for Stf0 in the kinetic analysis. In addition, these data make it possible to arrive at a reasonable hypothesis concerning the binding mode of PAPS and trehalose to Stf0. Detection of the Stf0–PAP–trehalose ternary complex confirmed two independent binding sites on Stf0 for the donor and acceptor substrates. Given the facts that the Stf0–PAPS binary complex was formed in the absence of trehalose and the Stf0–trehalose binary complex was formed in the absence of PAPS, a random, not ordered, manner of substrate binding and product release is clearly more favored. These findings suggest that a rapid equilibrium random sequential Bi-Bi mechanism is adopted by Stf0. As illustrated in Scheme 2, trehalose binds to both Stf0 and Stf0–PAPS binary complex, and PAPS binds to both Stf0 and Stf0–trehalose binary complex. The sulfuryl group is transferred from PAPS to trehalose inside the Stf0–PAPS–trehalose ternary complex, resulting in the formation of Stf0–PAP–T2S ternary complex. The two products are subsequently released from the enzyme active sites in a random mode, and the free enzyme, Stf0, is regenerated and continues to participate in the catalytic cycle. It is noteworthy that the intersection of the lines in Fig. 3 is on the horizontal axis (1/[PAPS]), which means that the binding of one substrate does not change the dissociation constant (K_d or K_m) for the other substrate in the rapid equilibrium random sequential Bi-Bi mechanism of Stf0.

Our findings on the catalytic mechanism of Stf0 are consistent with the recently solved X-ray crystal structure of Stf0 in the presence of trehalose [29]. As the first bacterial carbohydrate ST to be structurally characterized, Stf0 was found to share the most structural homology and conserved catalytic residues with Golgi-resident carbohydrate STs, including HNDST-1 and HS3OST-1 [21,22]. Based on the superposition of Stf0–trehalose complex structure with the structure of EST solved in the presence of PAPS and estradiol, an S_n2-like in-line displacement mechanism was postulated for Stf0 [29]. Our work further addresses the mechanistic details of Stf0, and the ternary complex formed in the proposed rapid equilibrium random sequential Bi-Bi mechanism for Stf0 supports the associative transition state in the in-line sulfuryl transfer mechanism as revealed by the structure.

Conclusions

Initial rate kinetics analyses, product inhibition study, and experiments in noncovalent complex identification have been used to delineate the catalytic mechanism of the bacterial carbohydrate sulfotransferase, Stf0. Rapid kinetic analyses of Stf0 were performed using an electrospray ionization mass spectrometry assay. This assay is particularly useful in characterizing enzymes for which no spectrophotometric assay is feasible. In addition to the compelling kinetic data, Stf0–PAPS and Stf0–trehalose binary complexes and Stf0–PAP–trehalose ternary complex were identified by FT-ICR mass spectrometry. Given both the kinetic and the noncovalent complex data, a rapid equilibrium random sequential Bi-Bi mechanism is evident for the catalysis of Stf0, and this result is consistent with the Stf0 structure data. The amino acid sequence in the catalytic active sites of Stf0 has been compared to that of cytosolic STs, Golgi-resident carbohydrate STs, and another bacterial carbohydrate ST, NodH. The conserved Arg^{15}, Ser^{152}, and Glu^{136} in the active sites of Stf0 are essential residues for the catalytic functions of Stf0, and the Ser is proposed to play a significant role in regulating the sequential mechanism. Our present study provides valuable information toward a comprehensive understanding of the structure, mechanism, and function of biologically important sulfotransferases using mass spectrometry.

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References

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