Clostridioides difficile TcdB Toxin Glucosylates Rho GTPase by an $S_{N}i$ Mechanism and Ion Pair Transition State

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ABSTRACT: Toxins TcdA and TcdB from Clostridioides difficile glucosylate human colon Rho GTPases. TcdA and TcdB glucosylation of RhoGTPases results in cytoskeletal changes, causing cell rounding and loss of intestinal integrity. Clostridial toxins TcdA and TcdB are proposed to catalyze glucosylation of Rho GTPases with retention of stereochemistry from UDP-glucose. We used kinetic isotope effects to analyze the mechanisms and transition-state structures of the glucohydrolase and glucosyltransferase activities of TcdB. TcdB catalyzes Rho GTPase glucosylation with retention of stereochemistry, while hydrolysis of UDP-glucose by TcdB causes inversion of stereochemistry. Kinetic analysis revealed TcdB glucosylation via the formation of a ternary complex with no intermediate, supporting an $S_{N}i$ mechanism with nucleophilic attack and leaving group departure occurring on the same face of the glucose ring. Kinetic isotope effects combined with quantum mechanical calculaations revealed that the transition states of both glucohydrolase and glucosyltransferase activities of TcdB are highly dissociative. Specifically, the TcdB glucosyltransferase reaction proceeds via an $S_{N}i$ mechanism with the formation of a distinct oxocarbenium phosphate ion pair transition state where the glycosidic bond to the UDP leaving group breaks prior to attack of the threonine nucleophile from Rho GTPase.

INTRODUCTION

Glycosyltransferases commonly catalyze glycosidic bond formation via the transfer of a sugar moiety from the activated nucleoside diphosphate sugar donor to acceptor substrates, including monosaccharides, polysaccharides, lipids, proteins, nucleic acids, and small organic molecules. Glycosyltransferases can be classified as inverting or retaining depending on the stereochemical outcome at the anomeric carbon. Inverting glycosyltransferases use a single-displacement mechanism ($S_{N}2$) with a single oxocarbenium ion-like transition state. The inverting mechanism has been supported by mechanistic studies and kinetic isotope effect measurements.

The reaction mechanism of retaining glycosyltransferases can occur by several proposed mechanisms (Figure 1). Initially, retaining glycosyltransferases were proposed to proceed through double-displacement mechanisms, where an active site amino acid acts as a nucleophile, attacking the anomeric carbon to form a covalently linked glycosyl-enzyme intermediate before the deprotonated acceptor nucleophile can attack, resulting in retained stereochemistry of the newly formed glycosidic bond (Figure 1a). However, retaining glycosyltransferases often lack a suitably positioned amino acid to act as the catalytic nucleophile. Retention of stereochemistry without a covalently linked glycosyl-enzyme intermediate led to the proposal for an “internal return” $S_{N}i$-like mechanism, where nucleophilic attack occurs at the same face as leaving group departure. The $S_{N}i$-like mechanism can proceed through a short-lived oxocarbenium ion intermediate (stepwise-mechanism) or a single oxocarbenium ion-like transition state (concerted mechanism) (Figure 1b, c).

KIE measurements have also been reported for trehalose-6-phosphate synthetase (OtsA), also supporting an $S_{N}i$ mechanism. However, the magnitude of the anomeric carbon KIE could not distinguish between stepwise and concerted $S_{N}i$ mechanisms. Recent quantum mechanics/molecular mechanics (QM/MM) studies have analyzed the retaining glycosyltransferases OtsA, α-1,2-mannosyltransferase, GalNAc-transferase, mannoglycercate synthetase, lipopolysaccharide α-galactosyl transferase, and glucosyl-3-phosphoglycerate synthetase.

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The TcdA and TcdB toxins from *Clostridoides difficile* contain a glucosyltransferase (GT) domain that when processed and released from endosomes into the cytosol of mammalian cells glucosylate and inactivate Rho GTPases.\(^\text{22−24}\) The TcdA and TcdB toxins are the primary determinants of *C. difficile* pathogenesis and as such are attractive therapeutic targets.\(^\text{23−28}\) Detailed knowledge of the reaction mechanism and transition states of TcdA and TcdB defines reaction chemistry and is useful in the design of transition state analogues. TcdA and TcdB catalyze transfer of a glucosyl residue to a specific threonine residue in the switch I effector regions of Rho GTPases, including Rac1. TcdA and TcdB belong to the GT44 family of glycosyltransferases, which also contains the bacterial toxin glucosyltransferases.\(^\text{29}\) The TcdA and TcdB glucosyltransferase domains belong to the GT-A fold family of glycosyltransferases. The TcdA and TcdB glucosyltransferase domains show the highest catalytic activity in the presence of a combination of potassium, magnesium, and manganese ions.\(^\text{30−32}\) Similar to other retaining glycosyltransferases, in the absence of an appropriately positioned acceptor to provide a catalytic nucleophile, the TcdA and TcdB toxins catalyze the slow hydrolysis of UDP-glucose.\(^\text{32,33}\)

Previous KIE measurements on the glucohydrolase reactions of TcdA and TcdB demonstrated a highly dissociative glucocationic transition state. This information was used to identify the iminosugars isofagomine and noeuromycin as transition state analogue inhibitors.\(^\text{33}\) Here, we characterize the mechanisms of both glucohydrolase (GH) and glucosyltransferase (GT) reactions of the TcdB GT domain. Retention of stereochemistry is confirmed for the TcdB GT reaction. In contrast, the TcdB GH reaction is demonstrated to proceed with the inversion of stereochemistry. Kinetic analysis of the TcdB GT reaction demonstrated that the reaction proceeds with the formation of a ternary complex. While positional isotope exchange analysis demonstrated that TcdB-catalyzed
glucosyltransfer occurs without reversible rotation of the leaving phosphoryl group.

KIE analysis combined with QM calculations support an S\textsubscript{N} mechanism with an oxocarbenium ion pair transition state for the TcdB GT reaction. Knowledge of the TcdB GH and GT transition states is useful for the design of transition analogues with improved potency to act as antitoxins.

## MATERIALS AND METHODS

### Gene Expression and Protein Purification

Expression plasmid pET28a containing C-terminal His-6 tagged TcdB-GTD (amino acids 1–546) was kindly provided by Tam et al.\textsuperscript{36} Conditions for the heterologous expression of TcdB-GTD in *E. coli* followed the method of Paparella et al.,\textsuperscript{35} with purification by Ni-NTA chromatography and protein preparation in 20 mM Tris pH 7.5, 150 mM NaCl, 15% v/v glycerol, freezing, and storage at −80 °C.

Expression of Rac1 GTPase (amino acids 1–192) also followed the method of Paparella et al.\textsuperscript{35} Expression from an open reading frame with an N-terminal His6 tag was cloned into pETDuet1 (Atum Bio). Rac1 GTPase was expressed in *E. coli* (One shot BL21 Star (DE3) cell line were adapted from Tam et al.\textsuperscript{36} and followed the method of Paparella et al.,\textsuperscript{35} with purification by Ni-NTA chromatography and protein preparation in 20 mM Tris pH 7.5, 150 mM NaCl, 15% v/v glycerol, freezing, and storage at −80 °C.

**Synthesis of Isotopically Labeled Substrates.** Isotopically labeled UDP-glucose was synthesized by coupled enzymatic reactions, as described previously.\textsuperscript{1} In summary, individual 1\textsuperscript{-H}, 2\textsuperscript{-H}, 6\textsuperscript{-H}, 1\textsuperscript{3}C, 1\textsuperscript{3}C, and 6\textsuperscript{13}C UDP-glucose were prepared by sequential actions of hexokinase, glucokinase, and inorganic pyrophosphatase. Isotopically labeled UDP-glucose were purified by Mono Q anion exchange HPLC in ammonium formate. Purity was established by coelution with commercially available UDP-glucose. 1\textsuperscript{18}O UDP-glucose and 1\textsuperscript{14}C, 1\textsuperscript{13}C, and 6\textsuperscript{13}C UDP-glucose were prepared by oxygen exchange at the anomeric carbon in H\textsuperscript{18}O (Cambridge isotope labs), detailed in the study of Paparella et al.\textsuperscript{35} Isotopic enrichment of 1\textsuperscript{14}O, 1\textsuperscript{13}C, and 6\textsuperscript{13}C UDP-glucose was assessed by mass spectrometry: Isotopic enrichment of 1\textsuperscript{14}O, 1\textsuperscript{13}C, and 6\textsuperscript{13}C double labeled UDP-glucose, the isotopic enrichment was assessed via mass spectrometry on a reaction without 1\textsuperscript{18}O that was run in parallel. Isotope enrichment was determined to be >95%. Mass spectra were acquired on a Shimazu LCMS-2010EV spectrometer.

**Stereochemistry of TcdB-GTD Glucosyltransferase Reaction.** The stereochemistry of the TcdB-GTD glucosyltransferase reaction was assessed by NMR spectroscopy. Rac1 was purified as described above and glucosylated by TcdB-GTD using 1\textsuperscript{13}C UDP-glucose. A glucosylation reaction was carried out for 90 min at room temperature and contained 50 mM HEPES pH 7.5, 100 mM KCl, 4 mM MgCl\textsubscript{2}, 1 mM MnCl\textsubscript{2}, 1 mM DTT, 200 μM 1\textsuperscript{13}C UDP-glucose, 160 μM Rac1, and 0.5 μM TcdB-GTD. After 90 min, the solution was concentrated to 500 μL using a 10 kDa Amicon spin column. D\textsubscript{2}O was added to a final concentration of 10% and transferred to a 5 mm NMR tube. The final concentration of Rac1 for NMR analysis was 280 μM. All NMR data were acquired at 25 °C with a Bruker AVIII 600 MHz spectrometer running TopSpin 3.6 and equipped with a 5 mm H/F-TCI cryogenic probe. A 2D 1\textsuperscript{13}C HSQC was acquired to characterize the products. The HSQC was run for 16 h with 512 scans acquired for each increment using a spectral width of 12 and 80 ppm for 1\textsuperscript{13}C and 1\textsuperscript{18}O, respectively, and a recycle delay of 1.5 s. The data sets were collected using 2048 and 32 complex points for 1\textsuperscript{13}C and 1\textsuperscript{18}O, respectively, and the time-domain NMR data were linearpredicted and zero-filled in the indirect dimension and multiplied with a shifted sine-bell function (SSB = 2) in each dimension prior to Fourier transformation.

**Kinetic Analysis of TcdB-GTD Glucosyltransferase Reaction.** Initial reaction rate experiments for TcdB-GTD glucosyltransferase activity were measured using 6\textsuperscript{13}C UDP-glucose as a substrate and by capturing radiolabeled glucosylated Rac1 protein by the precipitation of the protein product using the method described in the study of Bensadoun and Weinstein\textsuperscript{36} and Paparella et al.\textsuperscript{35} Initial reaction rate studies used fixed UDP-glucose/6\textsuperscript{13}C UDP-glucose or Rac1 at different concentrations while varying the concentrations of the other substrate. Terminated reaction samples were added to a precipitation mix of 120 μg/mL sodium deoxycholate, 6% trichloroacetic acid, and 10 μg/mL BSA. Samples were incubated at room temperature for 15 min before being centrifuged for 20 min at 13,800 rpm to separate the glucosylated protein product. The supernatant was removed, and the protein pellet was resuspended in 500 μL of 200 mM Tris–HCl pH 7.5, 5% SDS, and 20 mM NaOH. Samples were vortexed briefly and added to 20 mL scintillation vials to which 10 mL of Ultima Gold scintillation fluid was added (Perkin Elmer). The amount of glucosylated Rac1 was determined by scintillation counting of each sample for 1 min using a Tri-carb 2910TR scintillation counter (Perkin Elmer). The initial rates of the reaction were fit to the Michaelis Menten equation (eq 1), and a double-reciprocal Lineweaver–Burk plot was created. The data were best fit with a convergence of lines with a shared common x intercept.

**Positional Isotope Exchange Experiment.** Reaction mixtures contained 50 mM HEPES pH 7.5, 100 mM KCl, 4 mM MgCl\textsubscript{2}, 1 mM MnCl\textsubscript{2}, 1 mM DTT, 500 μM [1\textsuperscript{13}C\textsuperscript{1}\textsuperscript{8}O] UDP-glucose, 500 μM Rac1, and 0.1 μM TcdB-GTD. At 50, 100, 200, and 400 s, 150 μL aliquots of the reaction were added to 20 μL of 0.4 M EDTA (final concentration = 50 mM) to quench the reaction. This corresponded to 5, 20, and 40% conversion. Before NMR analysis, the reaction was diluted by 37% with D\textsubscript{2}O, TSP was added as an internal chemical shift reference, and samples placed into 3 mm NMR tubes. NMR data were acquired at 25 °C using a Bruker AVIII 600 MHz spectrometer running TopSpin 3.6 and equipped with a 5 mm H/F-TCI cryogenic probe. The anomeric cross peak of C1′ of UDP-glucose was determined in a high-resolution 2D 1\textsuperscript{13}C HSQC to resolve the cross-peak belonging to the C1′ bonded to 1\textsuperscript{8}O from that bonded to 1\textsuperscript{13}C expected to appear if positional isotope exchange (PIX) occurred during the enzymatic reaction. Each HSQC timepoint was run for 30 min with 8 scans acquired for each increment using a spectral width of 14 and 2 ppm for 1\textsuperscript{13}C and 1\textsuperscript{18}O, respectively, and a recycle delay of 1.1 s. The data sets were collected using 4096 and 128 complex points for 1\textsuperscript{13}C and 1\textsuperscript{18}O, respectively, and the time domain NMR data were linearpredicted in the indirect dimension and multiplied with a shifted sine-
bell function (SSB = 2) in each dimension prior to Fourier transformation. The extent of the enzyme reaction was quantitated by 2D $^1$H-$^13$C HSQC analysis of samples without enzymes and with enzymes to cause 40% reaction to quantitate the cross peak for glucosylated Rac1. Each HSQC was run for 9 h with 320 scans acquired for each increment using a spectral width of 12 and 80 ppm for $^1$H and $^{13}$C, respectively, and a recycle delay of 1.3 s. The data sets were collected using 4096 and 64 complex points for $^1$H and $^{13}$C, respectively, and the time-domain NMR data were linear-predicted in the indirect dimension and multiplied with a shifted sine-bell function (SSB = 2) in each dimension prior to Fourier transformation.

Attempted Glucocation Glucose in the TcdB-GTD Reaction. Reaction mixtures contained 50 mM HEPES pH 7.5, 100 mM KCl, 4 mM MgCl$_2$, 1 mM MnCl$_2$, 1 mM DTT, 300 μM [1-$^3$C] UDP-glucose, 500 μM Rac1, 5 μM TcdB-GTD, and 10% methanol. After reaction completion (2 h), proteins were removed by ultrafiltration (10 kDa Amicon spin column). The sample was made to 500 μl with 10% D$_2$O and transferred to a 5 mm NMR tube. NMR data were acquired as described for analysis of the TcdB-GTD glucosyltransferase reaction.

V/K KIEs for TcdB-GTD Glucosyltransferase Reaction. Measurements of V/K KIEs for the TcdB-GTD glucosyltransferase reaction were performed at room temperature using the competitive radiolabeled approach. Reaction conditions were as follows: 50 mM postassium phosphate, pH 6.0, 100 mM KCl, 4 mM MgCl$_2$, 1 mM MnCl$_2$, 1 mM EDTA pH 8.0, 50 μM [1-$^3$C] UDP-glucose, 500 μM Rac1, 5 μM TcdB-GTD, and 10% methanol. After reaction completion (2 h), proteins were removed by ultrafiltration (10 kDa Amicon spin column). The sample was made to 500 μl with 10% D$_2$O and transferred to a 5 mm NMR tube. NMR data were acquired as described for analysis of the TcdB-GTD glucosyltransferase reaction.

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$$r = \text{Channel A/Channel B}$$

(2)

Spectral deconvolution of the KIE data was achieved for $^3$H and $^{13}$C using eqs 3 and 4, respectively:

$$^3\text{H} = \text{Channel A} - \text{Channel B} \times r$$

(3)

$$^{13}\text{C} = \text{Channel B} + \text{Channel B} \times r$$

(4)

V/K KIE values were calculated from eq 5, where $R_0$ and $R_1$ are the ratios of ["heavy label"] UDP-glucose to ["remote label"] UDP-glucose prior to the reaction and at partial conversion, respectively, and $f$ is the fraction of substrate conversion for KIE measurements involving $^3$H,$^{13}$C, $^1$H$^{18}$O, and $^6$D$^{13}$C UDP-glucose:

$$\text{KIE}_{V/K} = \frac{\log(1 - f)}{\log(1 - f^{R_0/R_1})}$$

(5)

The observed KIE values were corrected for the $6^\circ$-$^3$H remote isotope effect using eq 6:

$$\text{KIE} = \text{KIE observed} \times 6 - ^3\text{H KIE}$$

(6)

TcdB-GTD Glucosyltransferase Forward Commitment. Forward commitment for UDP-glucose in the TcdB-GTD glucosyltransferase reaction was measured using the isotope trapping method. A 50 μl sample of the TcdB-GTD:UDP-glucose equilibrium complex was formed by incubating 5 μM TcdB-GTD with 70 μM $^6$-$^1$H UDP-glucose for 5 s in 50 mM phosphate pH 6.0, 100 mM KCl, 4 mM MgCl$_2$, 1 mM MnCl$_2$, and 1 mM DTT at room temperature. A solution of 450 μl of 5 mM unlabeled UDP-glucose, 30 μM Rac1, 50 mM phosphate buffer pH 6.0, 100 mM KCl, 4 mM MgCl$_2$, 1 mM MnCl$_2$, and 1 mM DTT) was rapidly mixed with the ES complex solution, and 4 × 50 μl aliquots were removed from the mixture and quenched with 50 μl of 100 mM EDTA pH 8.0 at 5, 10, 15, and 20 s after addition of chase solution. Control reactions containing no enzyme were processed in parallel to correct for background levels. Labeled glucosylated Rac1 protein was precipitated as described above. Spectroscopic counting was performed as described for KIE measurements. The amount of glucosylated Rac1 produced after the addition of chase solution was plotted as a function of time and extrapolated to time = 0. The concentration of the enzyme substrate (ES) complex was calculated using eq 7, where $E$ represents the enzyme concentration, $S$ represents the concentration of $^6$-$^1$H UDP-glucose, and $K_{eq}$ is the Michaelis constant for UDP-glucose in the TcdB-GTD glucosyltransferase reaction.

$$E = \frac{(ES)}{(S + K_{eq})}$$

(7)

$C_f$ was calculated using eq 8, where $Y$ is the ratio of moles of glucose product $P$ (y-intercept at $x$ = 0) per mole of the TcdB-GTD:UDP-glucose ES complex (eq 9).

$$C_f = \frac{Y}{(1 - Y)}$$

(8)

$$Y = \frac{P}{ES}$$

(9)

Solvent KIE. A solvent KIE for the TcdB-GTD glucosyltransferase activity was measured using the TcdB-GTD glucosyltransferase assay described above for V/K KIE measurements. Reactions were carried out in 0, 16, 32, 49, 65, and 82% D$_2$O. Glucosylated Rac1 was isolated using the protein precipitation method described above. Initial rates were used to calculate the solvent KIE using eq 10, where $k$ is the reaction rate, $n$ is the atom fraction of deuterium, and $C$ is commitment to catalysis. The intrinsic solvent KIE was calculated from the term $1/k_d^C$.

$$k_{d,0} = \frac{(1 + C)(1 - n + n\phi_k^f)}{1 + C(1 - n + n\phi_k^f)}$$

(10)

Computational Methods. Glucohydrolase and glucosyltransferase transition state structures for TcdB-GTD were determined from the experimentally measured intrinsic KIE values as constraints for theoretical transition state structures determined via density functional theory in Gaussian 09 using the B3LYP level of theory and the 6-31G(d) basis set. Calculated KIE values for theoretical transition state structures were calculated at 25 °C (TKELV = 298.15) using ISOEFP98 with scaled vibrational frequencies (SCFACT = 0.977) of optimized structures of UDP-glucose in the ground state and each transition state. For TcdB-GTD glucosyltransferase transition state calculations, a modified threonine acceptor nucleophile was included in optimized structures of the transition state. The input coordinates for the optimization of UDP-glucose in the ground state were taken from the x-ray crystal structure of the TcdA-GTD complex in complex with UDP-glucose (PDB ID: 3SRZ). The fully elaborated UDP-glucose structure was truncated to glucose-1-PO$_4^-$ and UDP-Mn$^{2+}$ and UDP-Mn$^{4+}$ leaving groups, resulting from the combined use of Mg$^{2+}$ and Mn$^{2+}$ in KIE experiments. Thus, the leaving group potential of the calculated and actual leaving groups should be noted. All structures were assigned a formal charge of 0 and a multiplicity of 1. The optimized ground-state structure was located as the global minimum and displayed no imaginary frequencies.

For the TcdB-GTD glucohydrolase reaction, initial transition state calculations were performed by fixing the 1–C–1–O(P) and 1–C–O(H$_2$O) bonds and varying their lengths from 1.8 to 3.0 Å in 0.2 Å
increments. Transition state structures were refined from the results of the first calculation by varying the 1–C–1–O(P) bond length from 2.2 to 2.4 Å in 0.01 Å increments while keeping the 1–C–O(H2O) bond length fixed at 2.2 Å. The final transition state structure had a single imaginary frequency. For the TcdB-GTD glucohydrolase reaction, a stepwise S\textit{i} mechanism was investigated, where the 1–C–1–O(P) bond was fixed at 3.0 Å and the 1–C–O(Thr) and 1–O(P)–H(Thr) bonds were fixed and varied from 1.8 to 3.0 Å in 0.2 Å increments. The final transition state structure was consistent with a distinct oxocarbenium ion intermediate. Equilibrium isotope effects (EIEs) for the intermediate were calculated with ISOEF98 using the optimized structure of the UDP-glucose ground state and the oxocarbenium ion. Electrostatic potential maps for the TcdB-GTD glucohydrolase and glucosyltransferase ground state and transition state structures were visualized in GaussView 6.0 (isovalue = 0.04) from the electron density and potential cubes acquired from the checkpoint files of the geometry optimization and frequency calculations.

## RESULTS AND DISCUSSION

### Stereochemistry of TcdB Glucohydrolase and Glucosyltransferase Reactions.

Transition state analysis of the TcdB glucosyltransferase domain (TcdB-GTD) was aided by the stereochemical outcome of the TcdB-GTD GH and GT reactions. We used 2D \textit{H}–\textit{13}C HSQC NMR spectroscopy to monitor the formation of \textit{α}-d-glucose or \textit{β}-d-glucose from TcdB catalyzed hydrolysis of UDP-glucose. However, d-glucose mutarotates between \textit{α}- and \textit{β}-anomeric forms with an equilibrium mixture of 36% \textit{α}-d-glucose and 64% \textit{β}-d-glucose at physiological pH values. The mutarotation of glucose in aqueous solution reaches equilibrium in approximately 2 hours.\textsuperscript{31–45} For \textit{α}-d-glucose and \textit{β}-d-glucose, the \textit{13}C–\textit{1}H anomeric peaks are found at approximately 5.3 and 4.7 ppm, respectively. TcdB-GTD (112.5 μM) and UDP-glucose (20 mM) were incubated at 25 °C, and NMR spectra were acquired 15 min after enzyme addition (approximately 90 turnovers). At this early time point, the anomeric carbon peak ratio corresponding to \textit{β}-d-glucose/\textit{α}-d-glucose = 3.9. Following equilibration of the glucose product at 180 min, the ratio stabilized at \textit{β}-d-glucose/\textit{α}-d-glucose = 1.4 (Figures 2a and S1). The TcdB-GTD GH reaction results in the formation of \textit{β}-d-glucose, establishing inversion of stereochemistry for TcdB-GTD GH activity.

Stereochemical outcome of the TcdB-GTD GT reaction was monitored by 2D \textit{1}H–\textit{13}C HSQC NMR for formation of the C1-H anomeric peak of the glucosylated Rho GTPase acceptor protein (Rac1). Based on earlier reports of the related C. sordellii lethal toxin, also a member of the large clostridial toxins, we anticipated TcdB-GTD to catalyze glucosylation of Rho GTPases with retention of stereochemistry.\textsuperscript{35,54} TcdB-GTD was incubated with Rac1 and [\textit{1–13}C glucose]UDP-glucose, and the C1-H anomeric peak corresponding to glucosylated threonine of Rac1 was monitored (Figure 2b). The peak corresponding to the C1-H anomeric peak of glucosylated Rac1 appeared at approximately 5.0 ppm, consistent with previous studies.\textsuperscript{44} As controls, we acquired spectra of \textit{α}-methyl-glucoside, \textit{β}-methyl-glucoside, and \textit{α}-glucosylated threonine. The overlay of the NMR spectra indicates \textit{α}-glucosylated threonine, thus confirming retention of stereochemistry for the TcdB-GTD GT reaction (Figure 2b).

### Kinetics of the TcdB Glucosyltransferase Reaction.

Retention of stereochemistry for the TcdB-GTD GT reaction implies a double-displacement mechanism or an \textit{S\textit{i}1} mechanism.

![Figure 2. NMR analysis of stereochemistry of TcdB-GTD glucohydrolase and glucosyltransferase reactions: (a) 2D \textit{1}H–\textit{13}C HSQC spectrum was enriched in \textit{β}-d-glucose early in the TcdB-GTD glucohydrolase reaction (blue) relative to mutarotation equilibrium (red). (b) 2D \textit{1}H–\textit{13}C HSQC spectrum for the C1′ anomeric carbon of glucosylated Rac1 formed in the TcdB-GTD glucosyltransferase reaction. The C1 and C1′ anomeric carbons of \textit{α}-glucose, \textit{β}-glucose, \textit{α}-methyl-glucoside, \textit{β}-methyl-glucoside, and \textit{α}-glucosylated threonine are indicated.](https://doi.org/10.1021/acschembio.2c00408)
oxocarbenium ion pair intermediate in the GT reaction of TcdB-GTD is tested by this PIX experiment. Double-labeled \([1\text{-}^{13}\text{C},\text{bridge-}^{18}\text{O}]\)UDP-glucose was synthesized to test for PIX in the TcdB reaction. The presence of the \(^{18}\text{O}\) label at the bridging position causes an upfield \(^{13}\text{C}\) NMR shift in the doublet from 98.33/98.38 to 98.30/98.35 ppm relative to \(^{16}\text{O}\) label at the bridging position (Figure 4c). The anomeric carbon is split into a doublet because it is coupled to the \(^{31}\text{P}\) via \(^{2}J\) (\(^{13}\text{C},\ ^{31}\text{P}\)) scalar coupling — \(^{31}\text{P}\) is spin 1/2, causing the \(^{13}\text{C}\) signal to split.

TcdB-GTD was incubated with excess \([1\text{-}^{13}\text{C},\text{1}\text{-}^{18}\text{O}]\)UDP-glucose and Rac1, and samples were quenched at 5, 20, and 40% conversion to UDP and glucosylated Rac1. For each sample, no shift in the UDP-glucose anomeric carbon peaks were detected (Figure 4), indicating that no PIX occurs for the TcdB-GTD GT reaction. Thus, the \(^{18}\text{O}\) label does not scramble from the bridging to the nonbridging position during the glucosyltransferase reaction.

A lack of PIX exchange establishes that the glucocation formed in the reaction chemistry is not free to permit \(\beta\)-phosphoryl rotation and reversible reaction to reform UDP-glucose. Chemical possibilities are that glucosyl loss is not complete prior to attack of the nucleophilic threonine of Rac1 and/or the \(\beta\)-phosphate of the UDP leaving group is restricted in the catalytic site and not free to rotate and/or that the glucocation transition state has low bond orders to both the leaving group and attacking nucleophile, its lifetime is too short to permit phosphoryl rotational equilibrium. This result is consistent with other retaining glycosyltransferases including galactosyltransferase,\(^{1}\) glycogen synthase,\(^{49}\) and sucrose synthetase,\(^{50}\) where no PIX effect has been reported.

**Attempted Glucocation Capture.** PIX analysis suggested a glucocationic transition state too sequestered to too short-lived to permit rotational equilibration of the \(\beta\)-phosphoryl group of UDP-glucose. An independent analysis of the presence of a stabilized glucocation is its reactivity (capture) by small nucleophiles. Capture of an oxocarbenium ion intermediate was attempted in the TcdB-GTD GT reaction.

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**Figure 3.** Steady state kinetic measurements on the TcdB-GTD glucosyltransferase reaction. Initial rates were fit to the Michaelis–Menten equation, and double reciprocal plots show lines intersecting on the x-axis. (a) Varying UDP-glucose concentrations at fixed Rac1 concentrations. (b) Varying Rac1 concentrations at fixed UDP-glucose concentrations.

**Figure 4.** Positional isotope exchange analysis of TcdB glucosyltransferase: (a) Positional isotope exchange reaction scheme. A glucocation with sufficient lifetime permits the weakly-bonded \(\beta\)-phosphoryl group of UDP-glucose to rotate and return to the reactant state with reposition of the bridging \(^{18}\text{O}\) to nonbridging \(^{18}\text{O}\) with a consequent NMR shift of the \(^{13}\text{C}\)-glucose anomeric carbon. (b) \(2D\ ^{1}\text{H}-^{13}\text{C}\) HSQC spectrum demonstrating the transfer of glucose from UDP-glucose to form glucosylated Rac1. Red is TcdB-GTD glucosyltransferase products at 40% completion and blue is the no enzyme control. c) \(2D\ ^{1}\text{H}-^{13}\text{C}\) HSQC spectrum showing the anomeric \(^{13}\text{C}\) carbon of \[^{13}\text{C}\text{-bridge-}^{18}\text{O}]\)UDP-glucose from the TcdB-GTD glucosyltransferase reaction quenched at 5% (blue), 20% (red), and 40% (green) completion. A TcdB-GTD glucosyltransferase reaction quenched at 40% completion (observed). As a PIX control for observation to nonbridge-\(^{18}\text{O}\), the \(2D\ ^{1}\text{H}-^{13}\text{C}\) HSQC spectrum of unlabeled UDP-glucose is compared (purple).
Table 2. Summary of V/K KIEs, Intrinsic KIEs and Calculated KIEs from QM Calculations for the TcdB-GTD Glucohydrolase Reaction

| label | type of KIE | experimental KIE TcdB-GTD | intrinsic KIE TcdB-GTD | calculated KIE TcdB-GTD |
|-------|-------------|--------------------------|------------------------|------------------------|
| $^1$$^3$H | α-secondary | 1.203 ± 0.001 | 1.216 ± 0.001 | 1.218 |
| $^1$$^3$C | primary label | 1.042 ± 0.002 | 1.045 ± 0.001 | 1.066 |
| $^1$$^3$O | primary leaving group | 1.026 ± 0.002 | 1.027 ± 0.002 | 1.025 |
| $^2$$^3$H | β-secondary | 1.012 ± 0.001 | 1.014 ± 0.001 | 1.014 |
| $^6$$^3$$^1$H | remote label | 1.053 ± 0.001 | 1.056 ± 0.001 | N/A |
| $^6$$^3$$^1$C | remote label | 1.000 | 1.000 | N/A |

*Positions of isotopic labels in the substrate UDP-glucose designed for multiple KIE measurement and the resulting experimental and theoretical KIEs. Colored labels correspond to those of Table 1. Experimental and intrinsic KIEs were previously measured and taken from ref 28. Six independent measurements were performed for each KIE, and the data represent the mean values ± SEM. Experimental KIEs were corrected for forward commitment (eq 7–9) to give intrinsic KIE values. The remote $^6$$^3$$^1$C value of unity provides the standard unity reference for all other isotope effects.

in the presence of 10% methanol. An oxocarbenium ion intermediate with lifetime and geometry to permit diffusional approach will react with methanol as a nucleophilic acceptor to form α-methyl or β-methyl glucose. No methyl-glucose was detected (Figures 2 and S2). Consistent with PIX, the TcdB-GTD GT reaction does not contain an oxocarbenium ion pair with a chemically reactive environment or reversible exchange.

**KIE on the TcdB Glucosyltransferase Reaction.** A glucosyltransfer $S_Ni$ mechanism can proceed through an oxocarbenium ion intermediate or a concerted mechanism with bond making to the Rac1 threonine simultaneous with loss of the UDP leaving group. We measured KIEs on the TcdB-GTD GT reaction to resolve these differences. KIEs were measured using the competitive radiolabeled approach with isotopically labeled UDP-glucose substrates. This method yields (V/K) KIEs, which report on all steps from substrate binding up to and including the first irreversible chemical step. For the TcdB-GTD GT reaction, departure of the UDP-glucose glycosidic bond at the transition state. The primary KIE reports on bonding and reaction coordinate motion of the anomeric carbon at the transition state. This isotope effect is typically 1.00–1.02 ($k_{light}/k_{heavy}$) for an $S_N1$ mechanism with a discrete oxocarbenium ion transition state, 1.025–1.06 for an $S_N2$-like concerted mechanism with partial bonds remaining to the anomeric carbon at the transition state, and 1.06–1.16 for an $S_N2$ associative concerted mechanism. The $[1$$^3$$^1$C] primary KIE was 1.010 ± 0.001, indicating the presence of an oxocarbenium ion pair between the anionic UDP leaving group oxygen and the cationic anomeric carbon, thereby supporting an $S_Ni$ mechanism with an oxocarbenium ion transition state.

The $[1$$^3$$^1$O] primary KIE reports on the extent of cleavage of the UDP-glucose glycosidic bond at the transition state. The $[1$$^3$$^1$O] KIE for the TcdB-GTD GT reaction was measured to be 1.037 ± 0.002. A fully cleaved glycosidic bond to generate an anionic leaving group is expected to generate a maximum KIE of 1.047. For TcdB-GTD, the KIE indicates substantial cleavage of the UDP-glucose glycosidic bond at the transition state. The magnitude of the TcdB-GTD $[1$$^3$$^1$O] KIE could also reflect full departure of the UDP leaving group with partial protonation of the departing oxygen, a transition state structure preceded by trehalose-6-phosphate synthetase (OtsA). Therefore, the $[1$$^3$$^1$O] KIE for the TcdB-GTD GT reaction is interpreted to reflect substantial leaving group bond cleavage with partial protonation of the leaving group oxygen.

The α-secondary $[1$$^3$$^1$H] KIE reports on the changes in bond hybridization at the anomeric carbon (sp vs sp$^2$) reflecting the degree of oxocarbenium ion character at the transition state. An sp$^2$ geometry at the transition state creates out-of-plane bending freedom of the hydrogen atom, generating a normal isotope effect. The $[1$$^3$$^1$H] KIE was measured to be 1.064 ± 0.003. Oxocarbenium ionlike transition states typically result in α-secondary KIEs of magnitude >1.15. In comparison, the $[1$$^3$$^1$H] KIE for the TcdB-GTD GH reaction was measured to be 1.216, and for the retaining glycosyltransferase OtsA, the α-secondary KIE
Figure 5. Optimized ground state and transition structures for TcdB glucohydrolase and glucosyltransferase reactions. (a) UDP-glucose structure is taken from PDB 3SRZ. The glycosidic bond is indicated by an asterisk. (b) Cut-off model for the ground state used to model the TcdB-GTD glucohydrolase transition state structure. Calculations were made by varying the lengths of the C−O bonds indicated by d1 and d2. (c) Calculated TcdB-GTD glucohydrolase transition state structure where calculated KIEs provided the best fit to experimental KIEs. (d) Cut-off model for the optimized ground state structure used to model the TcdB-GTD glucosyltransferase transition state. Calculations fixed the C−O bond (d1) at 3.0 Å and varied the lengths of the C−O bond and O−H bonds (d2 and d3, respectively). (e) Calculated TcdB-GTD glucosyltransferase transition state structure where the theoretical and intrinsic KIEs were in the best agreement.

is reported to be 1.284.16 The relatively small α-secondary KIE for the TcdB-GTD GT reaction could reflect a crowded environment at the transition state, where the out-of-plane bending mode that would normally occur for an sp² hydrogen atom is restricted by the encroaching Rac1 threonine acceptor nucleophile.

The β-secondary [2°,3-H] KIE reports on the degree of hyperconjugation between the σ(C−H) orbital at C2 to the σ*(C−O) orbital from the anomeric carbon to the UDP leaving group.53,56 The [2°,3-H] KIE for the TcdB-GTD GT reaction of 1.032 ± 0.002 suggests that the C2−H2 bond is near-perpendicular to the C1-UDP bond at the transition state.53 β-secondary KIEs of magnitude >1.07 have been reported for other N-glycohydrolases and glycosyltransferases with oxocarbenium ion-like transition states.16,53 As the primary [1°,14C] and [1°,18O] KIEs support an oxocarbenium ion-like transition state, the [2°,3-H] KIE reflects an unusual transition state geometry with little hyperconjugation from the σ(C−H) orbital at C2°. Oxocarbenium ion transition states are expected to generate C5-O5-C1-C2 atoms of the glucose ring in a near coplanar geometry. Four possible conformations of the hexopyranose ring can accommodate the co-planar arrangement, 2°,2B, 2°,2H4, and 2°,2H5 (Figure S5). Of the four possible structures, only the 2°,2H hexopyranose structure is consistent with the small β-secondary KIEs.12,57 The 2°,2H hexopyranose structure is likely to be a feature of the transition state geometry based on the small β-secondary [2°,3-H] KIE of (1.014) for the TcdB-GTD GH reaction.

A remote [6°,3-H] KIE was measured to be 1.071 ± 0.002 for the TcdB-GTD GT reaction. Remote tritium binding isotope effects contribute to V/K isotope effects and typically arise from enzyme-induced distortion of the sp³ geometry at C6°. This is a common feature among GH and GT enzymes. A similar remote tritium KIE was also observed for the TcdB-GTD GH reaction (Table 2).

The solvent deuterium KIE for the TcdB-GTD GT reaction was measured in the presence of varying amounts of D2O. The solvent KIE was calculated to be 2.24 ± 0.15 and a linear function of deuterium concentration. A single proton transfer occurring at the TcdB-GTD GT transition state is indicated (Figure S6).

Kinetic analysis and the KIEs support an S_{2,2} mechanism that occurs via an oxocarbenium ion pair transition state. Unusually small α-secondary and β-secondary KIEs are attributed to a B_{2,2} hexopyranose geometry and restricted environment for the ternary complex at the transition state.

Computational Analysis of the TcdB Glucohydrolase and Glucosyltransferase Transition States. Transition states for the TcdB-GTD GH and GT reactions were modeled using the experimental intrinsic KIEs as constraints for computational chemistry. Geometry for the transition state calculations used the initial coordinates for UDP-glucose (ground state) taken from a crystal structure of the TcdA GT domain in complex with UDP-glucose (PDB ID: 3SRZ). A simplified atomic cutoff model containing 29 atoms was used in the calculations. For optimization of the GH transition state structure, a water molecule was included on the β-face of glucose (32 atoms) (Figure Sb). Currently, there is no available structure of the ternary complex of TcdB-GTD in complex with UDP-glucose and an acceptor Rho GTPase protein. Although a structure of a TcdB-GTD:Cdc42:UDP complex has been recently published, glucose is absent during crystallization.18 Analysis of the GT transition state included a truncated nucleophilic
The theoretical transition states for the TcdB-GTD GH reaction were generated by varying the lengths of the C–O bond between the anomeric carbon and phosphate leaving group and the C–O bond between the anomeric carbon and incoming water nucleophile, marked as \(d_1\) and \(d_2\), respectively (Figure 5b). Theoretical KIEs for each potential transition state were calculated from scaled vibrational frequencies in ISOEEF98. Bond lengths from the anomeric carbon to the phosphate leaving group or to the incoming nucleophilic water were varied from 1.8–3.0 Å to find a theoretical transition state that would generate calculated KIEs to best match the experiment determinations (Table 2).

For the GH reaction, a theoretical transition state structure with C–O bond lengths of 2.33 and 2.20 Å to the phosphate leaving group and incoming water nucleophile, respectively, gave the best match between experimental and measured intrinsic KIEs (Figure 5c and Table 2). The \([{^1}\text{H}],{^{14}}\text{C}]\) calculated KIE was outside the experimental limits of the intrinsic KIE. However, experimental and calculated \([{^1}\text{H}],{^{14}}\text{C}]\) KIEs remains in agreement with a dissociative transition state with oxocarbenium ion character. The C5–O5–C1–C2 glucosyl atoms of the TcdB-GTD GH transition state are in a coplanar arrangement (Figure S7). The best-fit GH transition state from the family of KIEs predict the hexopyranose ring to be in the \(\text{H}_2\) half-chair conformation (Figures S5 and S7). The C4 atom is 0.1 Å above the plane on the \(\beta\)-face while the C3 atom is 0.6 Å below the plane on the \(\alpha\)-face. Only the small 2\(^{-}\)H \(\beta\)-secondary KIE is at variance with this transition state, which predicts a B\(_{2.5}\) transition state. It is possible that the geometry of the \(\beta\)-secondary C2\(^{-}\)H atom could be influenced by a strong transition state interaction with TcdB-GTD catalytic site amino acids.

Calculation of the TcdB-GTD GT transition state structure fixed the C–O bond between the anomeric carbon and phosphate leaving group at 3.0 Å for the test transition states (\(d_1\) in Figure 5d). This reflects full loss of the C1-UDP bond to permit initiation of nucleophilic attack from the same face by the Rac1 threonine. The C–O bond distances to the nucleophilic oxygen atom and for proton addition to the leaving group O atom were varied from 1.8–3.0 Å and 2.4–1.0 Å, respectively (\(d_2\) and \(d_3\) in Figure 5d). The computed GT transition state that best matched the \([{^1}\text{H}],{^{14}}\text{C}]\) and \([{^1}\text{H}],{^{18}}\text{O}]\) KIEs had C–O bond lengths of 3.0 Å to the anomeric carbon and an O–H bond length of 2.4 Å for protonation of the phosphate leaving group. Transition state geometry for the TcdB-GTD GT reaction supports an oxocarbenium ion pair at the transition state, causing the glucosyl C5–O5–C1–C2 atoms to be coplanar (Figure S7). The best-fit transition state from the family of KIEs places the hexopyranose ring in an envelope conformation (\(\text{E}\)), where the C4 atom is 0.7 Å above the plane on the \(\beta\)-face and the C3 atom is within the plane. Similar to the TcdB-GTD GH transition state, the small...
experimental $\alpha$ and $\beta$ secondary KIEs may result from a restricted active site when the UDP leaving group and Rac1 both crowd the same face of the anomeric carbon. These transition state structures were calculated without contributions from the active site amino acid interactions that may influence bond polarization.

Forward motion through the reaction coordinate for the GT reaction is supported by the PIX experiment, revealing an irreversible and/or restricted catalytic environment for the GT reaction, eliminating chemical reversal with torsional $\beta$-phosphoryl motion. The theoretical structure most closely related to the KIE constraints (Figure 5d) had imaginary frequencies along the O-H bond corresponding to proton transfer ($d_3$) and the C-O bond between the anomeric carbon and the nucophile oxygen ($d_2$) and is thus not a true transition state structure by the definition of a single imaginary frequency. Accordingly, equilibrium isotope effect (EIE) values were calculated. The intrinsic $[1^\text{″}-^\text{″}O]$ KIE matched a calculated EIE of 1.011 between UDP-glucose and the UDP + oxocarbenium ion, where the oxocarbenium ion has a lifetime that is adequate to permit bond vibrational modes to equilibrate. The intrinsic $[1^\text{″}-^\text{−}H]$ and $[2^\text{−}−^\text{″}H]$ KIEs also matched the calculated EIEs, consistent with a short-lived oxocarbenium ion intermediate. The $[1^\text{″}−^\text{−}C]$ calculated EIE was smaller than the experimental KIE, reflecting uncertainty in the degree of protonation for the leaving group oxygen atom.

Electrostatic potential surface (EPS) maps for the optimized TcdB-GTD ground state and GH and GT transition state structures indicate the distribution of partial positive (blue) and partial negative (red) charge (Figure 6). Key features of the TcdB-GTD GH and GT transition states center on the charge distribution around the cationic anomeric carbon of the glucose ring (Figure 6e-i). The positive charge on the anomeric carbon is most pronounced for the TcdB-GTD GT transition state (Figure 6i). Isofagomine, a 2,5 dideoxy-1-imino mimic of glucose, is a nanomolar inhibitor of the TcdA and TcdB toxins and binds only in the presence of UDP. The cationic isofagomine imino group forms a 2.6 Å ion pair with the $\beta$-phosphoryl group of UDP to mimic the transition state geometry. An EPS map from the crystal structure of isofagomine-TcdB was also generated (Figure 6i). The positive charge observed at the protonated endocyclic nitrogen atom of isofagomine is isosteric to the positively charged anomeric carbon in the TcdB-GTD GT transition state (Figure 6).

**CONCLUSIONS**

Organisms express several hundred glycosyltransferases, a handful of which have been characterized for transition state analysis. In contrast, humans express only three UDP-glucose based protein O-glycosyltransferases, and they are also rare in bacterial functions. Mechanistic and KIE analysis of the TcdB-GTD GT transition state expands mechanistic knowledge of the glycosyltransferases to the clinically important bacterial toxins. Knowledge of the transition states for TcdB-GTD glycosyltransferases has also provided the first generation of small molecule inhibitors.

Glucosylation of Rho GTPases by the TcdB toxin from C. difficile occurs with overall retention of stereochemistry and KIE measurements support an $S_{N}i$ mechanism with a distinct oxocarbenium phosphate ion pair transition state. The glucocation complex has a lifetime long enough to conform to EIE and thus shares characteristics of an intermediate. However, the complex is too transient or too constrained to permit torsional equilibration of the UDP-phosphoryl group. Combined KIE and QM calculations demonstrate that the glycosidic bond to the leaving group is cleaved before the approach of the nucleophilic threonine and that the distances from the anomeric carbon to the leaving group and the nucleophile are large (3.0 Å), indicating the presence of an oxocarbenium ion pair. The TcdB glucosyltransferase is therefore similar to some other retaining glycosyltransferases reported to react by an $S_{N}i$ mechanism and ion pair transition states. Enzymes forming glucocation-like transition states are susceptible to inhibition by iminosugar analogues that mimic the cationic nature of the transition state. Thus, isofagomine and neoeurymycin mimic the distinct oxocarbenium ion present in the TcdB-GTD GT transition state and are nanomolar inhibitors of the enzyme. The transition state structure of the TcdB glycocyltransferase domain will be useful in designing second-generation transition state analogues toward potential therapeutics for C. difficile infections.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.2c00408.

NMR analysis of stereochemistry of TcdB-GTD glucosylhydrolyase reaction; NMR analysis of TcdB-GTD glucosyltransferase reaction in the presence of 10% methanol; forward commitment (Cl) for TcdB-GTD glucosyltransferase activity; summary of intrinsic KIEs for TcdB-GTD glucosyltransferase reaction and glucosylhydrolyase reaction; conformations for a glucocation; proton inventories for TcdB-GTD glucosyltransferase reaction; calculated TcdB-GTD glucosylhydrolyase and glucosyltransferase transition state structures; and atomic coordinates for QM transition state analysis (PDF)

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**Author Contributions**

A.S.P. performed synthesis of UDP-glucose substrates, preparation of all NMR samples, KIE analysis of TcdB-GTD...
catalyzed reaction, and QM calculations on TcDB-GTD catalyzed reactions. S.M.C. acquired and helped to analyze all NMR data. B.L.A. performed kinetic analysis of TcDb-GTD glucosyltransferase reaction and performed Lineweaver–Burk analysis. V.L.S. designed and supervised the project. All authors contributed to writing the manuscript. All authors have given approval to the final version of the manuscript.

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Notes
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ABBREVIATIONS
GT glucosyltransferase
GH glucohydrolase
KIE kinetic isotope effect
QM quantum mechanics
EIE equilibrium isotope effect.

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