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Mutational Effects on Transglycosylating Activity of Family 18 Chitinases and Construction of a Hypertransglycosylating Mutant

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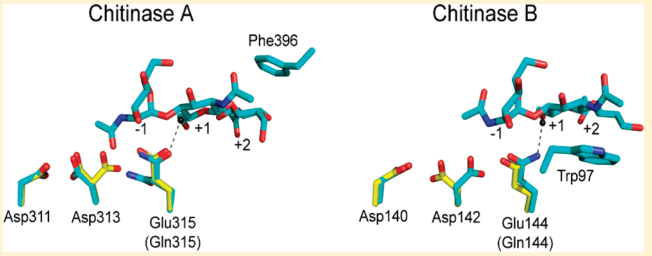
Supporting Information

ABSTRACT: Enzymatic features that determine transglycosylating activity have been investigated through site-directed mutagenesis studies on two family 18 chitinases, ChiA and ChiB from *Serratia marcescens*, with inherently little transglycosylation activity. The activity was monitored for the natural substrate $(GlcNAc)_4$ using mass spectrometry and HPLC. Mutation of the middle Asp in the diagnostic DxDxE motif, which interacts with the catalytic Glu during the catalytic cycle, yielded the strongly transglycosylating mutants ChiA-D313N and ChiB-D142N, respectively. Mutation of the same $Asp^{313/142}$ to Ala or the mutation of $Asp^{311/140}$ to either Asn or Ala had no or much smaller effects on transglycosylating activity. Mutation of Phe^{396} in the +2 subsite of ChiA-D313N to Trp led to a severalfold increase in transglycosylation rate while replacement of aromatic residues with Ala in the aglycon (sugar acceptor-binding) subsites of ChiA-D313N and ChiB-D142N led to a clear reduction in transglycosylating activity. Taken together, these results show that the transglycosylation properties of family 18 chitinases may be manipulated by mutations that affect the configuration of the catalytic machinery and the affinity for sugar acceptors. The hypertransglycosylating mutant ChiA-D313N−F396W may find applications for synthetic purposes.



Oligosaccharides of specific compositions and length have considerable potential, for example as prebiotics,[1−3] therapeutics,[4−8] or in agriculture-related applications.[9−11] Production of specific oligosaccharides by chemical methods is challenging due to the need to selectively protect and manipulate chemically quite similar saccharide donors and acceptors. For this reason, development of methods for enzymatic synthesis of oligosaccharides is desirable.

One of the enzymatic alternatives comprises the use of glycoside hydrolases. Besides hydrolysis of glycosidic bonds, many of these enzymes are able to catalyze transglycosylation reactions to form new glycosidic bonds between donor and acceptor saccharides.[12−16] In retaining glycoside hydrolases, the transglycosylation reaction occurs through a double-displacement mechanism.[17] In the first step the glycosidic oxygen is protonated by a catalytic acid, and the anomeric carbon is a target for a nucleophilic attack from the catalytic base, leading to cleavage of the glycosidic bond and to formation of a glycosyl−enzyme intermediate. In the second step, the intermediate decomposes with different possible outcomes. Hydrolysis is attained by attack of a water molecule on the glycosyl−enzyme intermediate that is assisted by the conjugated base of the catalytic acid residue and that leads to release of a hydrolyzed sugar. Alternatively, transglycosylation may occur if the water molecule is outcompeted by another acceptor such as a carbohydrate or an alcohol. Since transglycosylation is a kinetically controlled reaction, efficient transglycoslation requires an enzyme with an active site architecture that disfavors correct positioning of the hydrolytic water molecule and/or favors binding of incoming carbohydrate molecules, through strong interactions in the aglycon subsites.[18]

The transglycosylation activity of family 18 chitinases[19−23] is of special interest because there are numerous potential applications for chitooligosaccharides [i.e., homo- or hetero-oligomers of glucosamine (GlcN)[1] and N-acetylated glucosamine (GlcNAc)], especially in the food, medical, and agriculture fields.[6,24] Examples of biological activity for chitooligosaccharides include antifungal activities,[25] antitumor properties,[7] antibacterial activities,[26] and immunoenhancing effect.[27−29] The bioactivities of chitooligosaccharides are thought to depend on a combination of oligomer length, degree of acetylation, and acetylation pattern (sequence).[30] Transglycosylating chitinases have the potential to play a central role in the development of new well-defined mixtures of chitooligosaccharides with new or improved biological activity, by coupling smaller chitooligosaccharide building blocks to each other or to other functional groups.

Family 18 chitinases are retaining glycoside hydrolases that are special in the sense that the nucleophilic attack is carried out by the acetamido group of the sugar bound to the −1 subsite (Figure 1). Consequently, the intermediate is not a covalent glycosyl−enzyme intermediate, but a noncovalent oxazolinium ion (Figure 1). The positioning and nucleophilicity of the acetamido group are heavily affected by residues on the enzyme,

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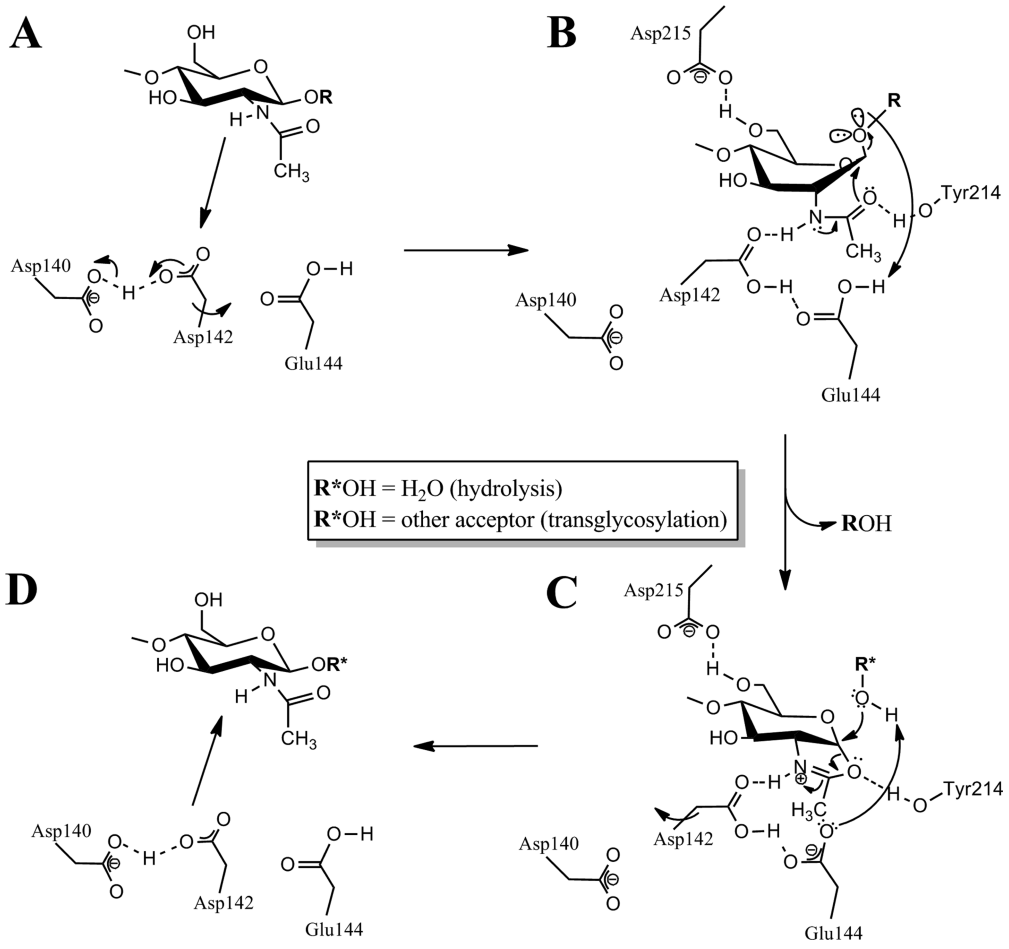

Figure 1. Catalytic mechanism of family 18 chitinases exemplified with ChiB from *S. marcescens*. The figure is modified from ref 33. See text for details. (A) Substrate binds the resting enzyme and induces rotation of Asp^{142} toward Glu^{144}. (B + C) Substrate binding causes distortion of the −1 sugar to a skew-boat conformation (only the −1 sugar is shown); Glu^{144} functions as a general acid and facilitates leaving group departure by protonating the glycosidic oxygen. Simultaneously, a nucleophilic attack by the acetamido group of the −1 sugar also promotes leaving group departure, leading to the formation of an oxazolinium ion intermediate (C). (C + D) Finally, Glu^{144} acts as a general base and activates an acceptor molecule that attacks the oxazolinium ion. This results in a hydrolytic or a transglycosylation product with retained conformation at the anomeric carbon (D).

in particular by a conserved aspartate (Asp^{142} in Figure 1) and tyrosine (Tyr^{214} in Figure 1).[31−33] Asp^{142} is part of the fully conserved diagnostic DxDxE motif that includes the catalytic acid (Glu^{144} in Figure 1) and another aspartate (Asp^{140} in Figure 1) at a relatively buried position in the core of the $(\beta/\alpha)_8$ (TIM-barrel) fold, which is considered the catalytic domain in family 18 chitinases (see legend to Figure 2). Crystallographic studies of wild-type and mutant family 18 chitinases have shown that Asp^{142} may occur in two positions: "up", interacting with Glu^{144}, or "down", interacting with Asp^{140} (Figure 2 and refs 33−37). Studies on Chitinase B (ChiB) from *Serratia marcescens* have shown that the charged and partly buried Asp^{140} is stabilized by hydrogen-bonding interactions with Ser^{93} and Tyr^{10} in conformations where the protonated Asp^{142} (Figure 1) is in the up position.[32,33] It has been suggested that rotation of Asp^{142} and the accompanying adjustments around Asp^{140} are essential elements of the catalytic cycle as shown in Figure 1. Asp^{142} affects the stability of the oxazolinium ion intermediate and the pK_a of the catalytic glutamate that needs to "cycle" during catalysis due to the dual role of this residue as acid in the glycosylation step and as water-positioning and -activating base in hydrolysis of the oxazolinium ion (Figure 1 and refs 32 and 33). It is impossible to predict how exactly changes in the "Asp^{140}-Asp^{142} part" of the catalytic machinery affect the stability of the oxazolinium ion and the reactivity of the catalytic water. However, on the basis of the above considerations, we considered it quite conceivable that mutation of Asp^{142} or residues affecting Asp^{142} could affect the transglycosylation activity.

Even though transglycosylation is a widespread phenomenon in family 18 chitinases, there is little understanding of the structural basis underlying this activity. The suggestion that subtle variations in the catalytic machinery may play a role is supported by structural and functional analysis of the W167A mutant of Chitinase A from *S. marcescens* (ChiA).[34] While this mutant, which carries a mutation in a donor subsite (−3), has reduced affinity for carbohydrate substrates,[38,39] it shows increased transglycosylation activity. Aronson et al.[34] ascribe this to changes in the position of Asp^{313} (analogous to Asp^{142}, above) that they observed by X-ray crystallographic analysis. The roles of aromatic residues potentially involved in binding of sugar acceptors has been addressed in several studies showing that these residues indeed contribute substantially to substrate binding.[40−44] Very recently, it has been demonstrated that removal of aromatic residues in acceptor subsites reduces transglycosylation activity,[23,45] providing evidence for the importance of acceptor affinity for transglycosylating activity.

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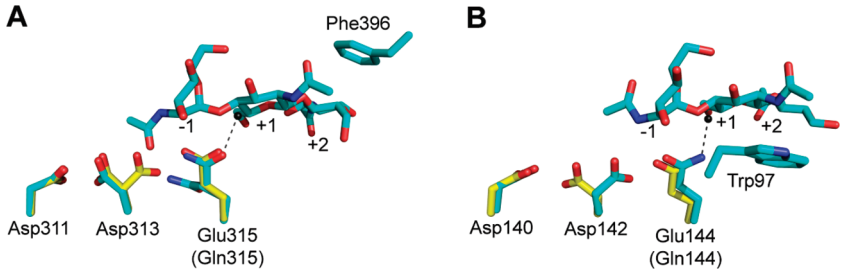

Figure 2. Overview of residues mutated in this study in ChiA (A) and ChiB (B). (A) superposition of ligand-free wild-type ChiA (yellow carbon atoms; only the catalytic triad Asp^{311}-Asp^{313}-Glu^{315} is shown; PDB accession code 1EDQ),[36] ChiA-E315Q in complex with $(GlcNAc)_8$ (blue carbon atoms; only three GlcNAc units are shown as well as the catalytic triad and the side chain of Phe^{396}; PDB accession code 1EHN)[36] and ChiA in complex with the reaction intermediate analogue allosamidin (only one water molecule shown as gray sphere; PDB accession code 1FFQ).[62] (B) superposition of ligand-free wild-type ChiB (yellow carbon atoms; only the catalytic triad Asp^{140}-Asp^{142}-Glu^{144} is shown PDB accession code 1E15),[57] ChiB-E144Q in complex with $(GlcNAc)_5$ (blue carbon atoms; only three GlcNAc units are shown as well as the catalytic triad and the side chain of Trp^{97}; PDB accession code 1E6N)[33] and ChiB in complex with allosamidin (only one water molecule shown as gray sphere; PDB accession code 1E6R).[33] Nitrogen atoms are colored dark blue, and oxygen atoms are colored red. Hatched lines indicate hydrogen bonds. The numbers indicate binding subsites, numbered according to Davies et al.[63] and Biely et al.[64] The water molecule taken from the allosamidin complex structures is thought to be located approximately as the catalytic water would be and forms hydrogen bonds with Oε1 of $Glu^{315/144}$ and two other water molecules (not shown). ChiA and ChiB consist of two domains: a catalytic domain, constituting of a $(\beta/\alpha)_8$ (TIM-barrel) fold and an $\alpha + \beta$ insert (N-terminal in ChiA and C-terminal in ChiB), and a chitin-binding domain (C-terminal in ChiA and N-terminal in ChiB).[57,58] The aspartates and glutamate shown in the figure represent the residues in the diagnostic DxDxE motif that spans strand 4 of the TIM-barrel. Note that in the structures of ligand-free and ligand-bound ChiA enzyme, Asp^{313} and Gln^{315}, respectively, were found to be equally distributed between two orientations. This figure was made using PyMol (DeLano Scientific).

Table 1. Kinetic Constants of ChiAwt, ChiBwt, and Mutants for the Hydrolysis of $(GlcNAc)_4$ in 20 mM Sodium Acetate, pH 6.1, and 0.1 mg/mL BSA at 37 °C[a]

enzyme	k_{cat} (s^{-1})	K_m (μM)	relative k_{cat}/K_m (%)	transglyc activity[b]
		Chitinase A		
ChiAwt	33 ± 1[c]	9 ± 1[c]	100[c,d]	+
D311A	0.021 ± 0.001	10 ± 1	0.057	+
D311N	0.032 ± 0.003	36 ± 6	0.024	+
D313A	0.032 ± 0.003	82 ± 23	0.011	+
D313N	0.60 ± 0.07	3.4 ± 1.3	4.87	+++
D313N/F396A	0.024 ± 0.002	18 ± 5	0.035	++
D313N/F396W	0.14 ± 0.01	2.7 ± 0.6	1.38	+++
		Chitinase B		
ChiBwt	28 ± 2[c]	4 ± 2[c]	100[c,d]	−
D140A	0.12 ± 0.01	23 ± 3	0.074	++
D140N	0.26 ± 0.03	79 ± 24	0.047	−
D142A	0.009 ± 0.001	13 ± 4[e]	0.01	−
D142N	0.25 ± 0.01	1.0 ± 0.3[f]	3.57	+++
D142N/W97A	0.89 ± 0.02	77 ± 6	0.17	++

[a] The data used for calculation of the kinetic constants are shown in Figures S1−S13 of the Supporting Information. [b] +++: high activity; ++: medium activity; +: minimal activity; −: no activity. [c] Data from Krokeide et al.[49] [d] Absolute k_{cat}/K_m values for the wild-type enzymes are 3.7 μM s^{-1} (ChiAwt) and 7.0 μM s^{-1} (ChiBwt). [e] There is an inherent uncertainty in this value. Because of the low activity of ChiB-D142A, enzyme concentration of 3 μM was used to have significant hydrolysis with the time scale of the experiment. [f] Because of a normal detection threshold of 2 μM in the experimental setup used, this value has an inherent uncertainty and should be viewed as a maximum value for ChiB-D142N.

In the present study, we have addressed both of the two factors that a priori are thought to affect transglycosylation activity using a site-directed mutagenesis approach. We have studied the effects of mutating $Asp^{140/311}$ and $Asp^{142/313}$ (analogous residues in ChiB and ChiA from *S. marcescens*, respectively), expecting that these mutations could affect the stability of the oxazolinium ion intermediate and the reactivity of the catalytic water. In addition, we have probed the roles of aromatic residues in the aglycon subsites since these are likely to affect the affinity for carbohydrate acceptors. The latter part of the study not only includes mutants with reduced acceptor affinity showing reduced transglycosylation activity but also a rationally designed mutant with increased acceptor affinity that shows increased transglycosylation activity.

EXPERIMENTAL PROCEDURES

Enzymes. The Chitinase genes *chia* and *chib* from *S. marcescens* strain BJL200 were expressed in *Escherichia coli* (Invitrogen) under control of their own promoter.[46,47] Mutagenesis was performed

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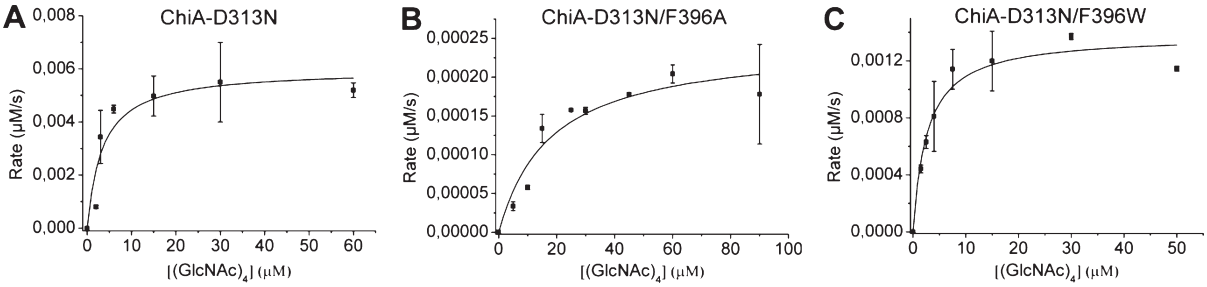

Figure 3. Michaelis−Menten plots for hydrolysis of $(GlcNAc)_4$ with (A) 10 nM ChiA-D313N, (B) 10 nM ChiA-D313N/F396A, and (C) 10 nM ChiA-D313N/F396W at pH 6.1 and 37 °C. Solid symbols are experimentally determined data for the rate of $(GlcNAc)_4$ hydrolysis (as assessed by the appearance of $(GlcNAc)_2$, the absolutely dominant product observed under these reaction conditions), while the solid lines represent the best nonlinear fit using the Michaelis−Menten equation. The error bars represent the standard deviations from two individual experiments.

using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) essentially as described by the manufacturer. The primers used for the mutagenesis are listed in Table S1 of the Supporting Information. To confirm that the *chia* and *chib* genes contained the desired mutations and to check for the occurrence of nondesirable mutations, the mutated genes were sequenced using an ABI PRISM dye terminator cycle sequencing ready reaction kit and an ABI PRISM 377 DNA sequencer (PerkinElmer Life Sciences). Wild-type and mutant chitinases were overexpressed in *E. coli* and purified as described elsewhere.[48] Enzyme purity was verified by SDS-PAGE and estimated to be >95% in all cases. Protein concentrations were determined by using the Quant-IT protein assay kit and Qubit fluorometer from Invitrogen.

Chromatography (HPLC) of Chitooligosaccharides. Chitooligosaccharides (Seikagaku Corp., Tokyo, Japan) were separated with regard to size by normal-phase HPLC using a Tosoh TSK Amide-80 column (a 0.46 × 25 cm column was used in all analyses with the exception of the kinetic analyses, where a 0.20 × 25 cm column was used) with an Amide-80 guard column. The sample size was 10 μL, and the chitooligosaccharides were eluted isocratically at 0.25 mL/min with 70% (v/v) acetonitrile at 22 °C. The chitooligosaccharides were monitored by measuring absorbance at 210 nm and quantified by comparing peak areas with peak areas obtained from samples with known concentrations. Using these standard samples, it was established that there was a linear correlation between peak area and oligosaccharide concentration within the concentration range used in this study.

Kinetic Analysis. The kinetic constants k_{cat} and K_m of the ChiA and ChiB mutants were determined using the $(GlcNAc)_4$ substrate,[49] which, at the substrate concentrations and in the time frames used for kinetic analysis, is predominantly (>90% for ChiA; close to 100% for ChiB) hydrolyzed into two dimers by all enzyme variants in this study. Reaction mixtures with 5−10 different $(GlcNAc)_4$ concentrations varying from 1.5 to 1000 μM (see Figures S1−S6 and S8−S12 of the Supporting Information), in 20 mM sodium acetate buffer, pH 6.1 and 0.1 mg/mL BSA (final concentrations), were preincubated in a 37 °C water bath for 10 min before the reactions were started by adding purified enzyme to reach the desired enzyme concentration and a total reaction volume of 1.0 mL. Final enzyme concentrations were as follows: ChiA-D311A, 0.7 μM; ChiA-D311N, 0.5 μM; ChiA-D313A, 2.0 μM; ChiA-D313N, 10 nM; ChiA-D313N/F396A, 10 nM; ChiA-D313N/F396W, 10 nM; ChiB-D140A, 0.25 μM; ChiB-D140N, 0.25 μM; ChiB-D142A, 3 μM; ChiB-D142N, 35 nM; ChiB-D142N/W97A, 10 nM. To determine the rate of $(GlcNAc)_4$ hydrolysis at a specific concentration, seven samples of 50 μL, including one at time equal zero, were withdrawn at regular time intervals over a total period of 2−45 min, and the enzyme was inactivated by adding 50 μL of acetonitrile. All of the reactions were run in duplicate, and all of the samples were stored at −20 °C until HPLC analysis. Reaction conditions were such that the rate of hydrolysis of $(GlcNAc)_4$ was essentially constant over time, with the $(GlcNAc)_4$ concentration always staying above 80% of the starting concentration. Data points were only discarded when hydrolysis had not taken place or more than 20% of the initial $(GlcNAc)_4$ were hydrolyzed (to ensure initial rates only). If more than two data points, out of the seven, had to be removed due to the reasons described above, the whole set was discarded. The slopes of plots of 0.5 times the $(GlcNAc)_2$ concentration versus time were taken as the hydrolysis rate. The rates were plotted versus substrate concentration in a Michaelis−Menten plot, and the experimental data were fitted to the Michaelis−Menten equation by nonlinear regression using the Origin v7.0 (OriginLab Corp., Northampton, MA).

Transglycosylation Assay with the $(GlcNAc)_4$ Substrate. 1.0 mM $(GlcNAc)_4$ was incubated with 3.0 μM Chitinase in 20 mM ammonium acetate, pH 6.1, and 0.1 mg/mL BSA (final concentrations) in a water bath at 37 °C. Samples of 50 μL were withdrawn at regular time intervals, and the Chitinase was inactivated by adding 50 μL of acetonitrile. All of the reactions were run in duplicate, and all of the samples were stored at −20 °C until HPLC analysis of reaction products, as described above.

Some of the samples were also analyzed by mass spectrometry. 1 μL samples were withdrawn from the reaction mixture and mixed with 2 μL of a matrix solution (15 mg/mL 2,5-dihydroxybenzoic acid, in 30% acetonitrile) and spotted directly on a target plate for MALDI-TOF MS analysis. The spotted samples were immediately dried using a heat gun. MS spectra of the products were acquired using an Ultraflex TOF/TOF MS (Bruker Daltonik GmbH, Bremen, Germany) with gridless ion optics under control of Flexcontrol 4.1. The experiments were conducted using an accelerating potential of 24 kV in the reflector mode.

$(GlcNAc)_6$ and $(GlcNAc)_3$ Hydrolysis. 150 μM of $(GlcNAc)_6$ was incubated with Chitinase in 20 mM ammonium acetate, pH 6.1, and 0.1 mg/mL BSA (final concentrations) in a 37 °C water bath. The enzyme concentrations were 300 nM, except for ChiA-D313N (70 nM), ChiA-D313N/F396W (100 nM), and ChiA-D313A (500 nM). The $(GlcNAc)_3$ reactions were performed under similar conditions, but with a substrate and enzyme concentration of 1 mM and 3 μM, respectively. Samples were

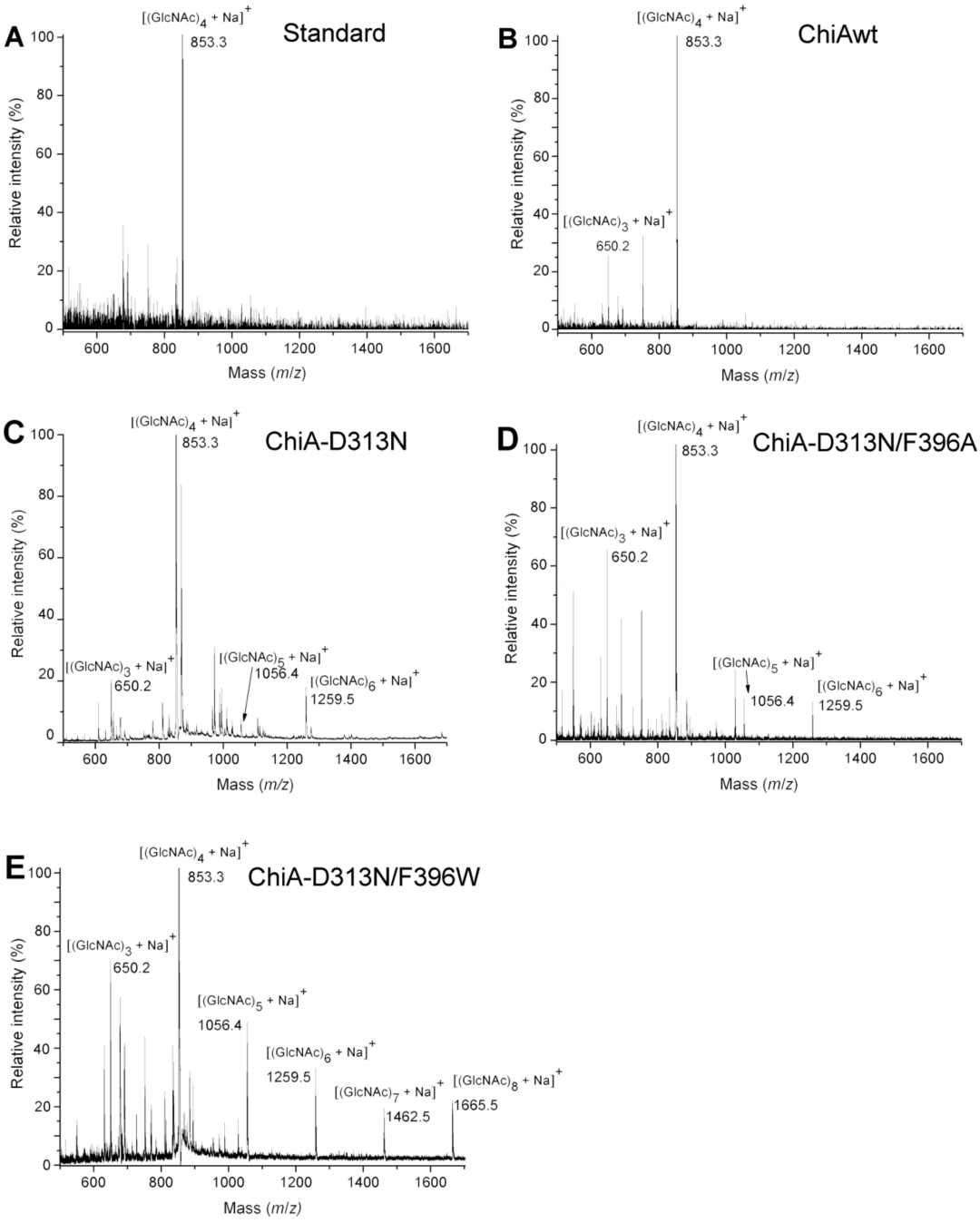

Figure 4. MALDI-TOF MS analysis of (A) 1.0 mM $(GlcNAc)_4$ standard sample and of reaction mixtures containing 1.0 mM $(GlcNAc)_4$ and 3.0 μM ChiAwt (B), ChiA-D313N (C), ChiA-D313N/F396A (D), or ChiA-D313N/F396W (E) in 20 mM ammonium acetate (0.1 mg/mL BSA, pH 6.1) after 20 s (ChiAwt), 5 min (ChiA-D313N/F396A and ChiA-D313N/F396W) or 6 min (ChiA-D313N) incubation at 37.0 °C.

quenched and analyzed by HPLC as described above. The data were used to determine specific activities toward $(GlcNAc)_{6,3}$ [derived from the (linear) slopes of the plots of the $(GlcNAc)_{6,3}$ concentration versus time] and for $(GlcNAc)_6$ to estimate cleavage preferences for this substrate (i.e., producing two trimers or producing a dimer and a tetramer).

RESULTS

Mutant Design and Initial Mutant Characterization. To investigate the effect the aspartates in the DxDxE motif have on transglycosylation activity, we have mutated Asp^{311} and Asp^{313} in ChiA and the corresponding Asp^{140} and Asp^{142} in ChiB to Asn or Ala. With respect to aromatic residues, we chose to focus on Phe^{396} in the +2 subsite for ChiA and Trp^{97} in the +1 subsite for ChiB. In ChiA, the single point mutation Asp to Ala in position 313 (ChiA-D313N) was combined with a Phe to Trp or Ala exchange, respectively (ChiA-D313N/F396W and ChiA-D313N/F396A). The Phe → Trp exchange in ChiA allows for an increase in the aromatic surface area that incoming sugar acceptor can interact with while the Phe → Ala completely removes this area. In ChiB, the single point mutation Asp to Ala in position 142 (ChiB-

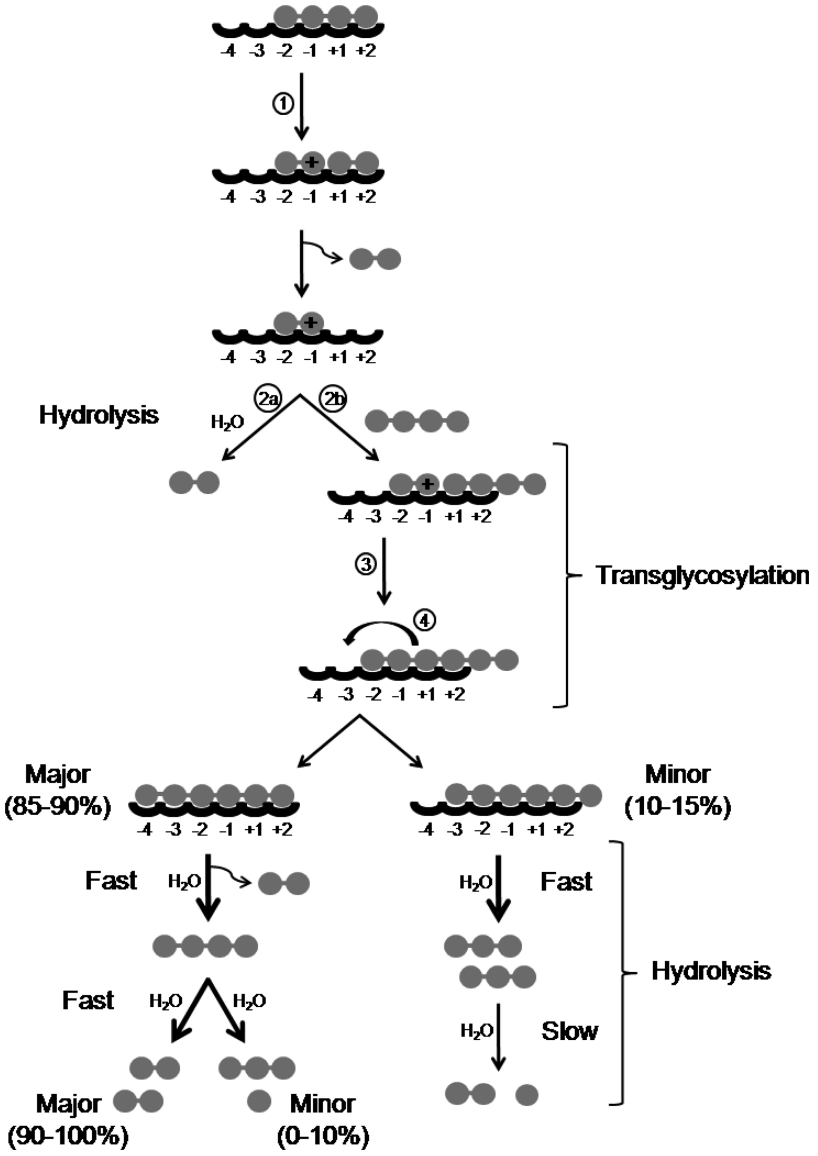

Figure 5. Schematic depiction of a possible transglycosylation reaction using $(GlcNAc)_4$ as substrate. GlcNAc residues are shown as gray circles, and the binding subsites of the enzyme (here; ChiA) are numbered. The $(GlcNAc)_4$ substrate binds to the −2 to +2 subsites, and the glycosidic bond is cleaved to form a positive charged "$(GlcNAc)_2$" oxazolinium ion intermediate (marked with +) in the −1 and −2 subsites (step 1). The $(GlcNAc)_2$ aglycon product then exits and is replaced by an incoming water (step 2a) or acceptor $(GlcNAc)_4$ (step 2b). In the second case (transglycosylation), the O4 of the nonreducing-end GlcNAc of $(GlcNAc)_4$ completes the reaction by nucleophilic attack on C1 of the glycosyl in the −1 site and forms $(GlcNAc)_6$ (step 3). This transglycosylation product is released and may after rebinding (step 4) be subjected to hydrolysis or further transglycosylation reactions. Further transglycosylation may lead to production of longer oligosaccharides of "any" length (see also Figure 4). These longer oligomers will be good substrates, and they will eventually be converted to trimers and dimers by hydrolysis but may yield novel tetramers along the way.

D142N) was combined with a Trp to Ala exchange (ChiB-D142N/W97A) to remove the aromatic surface area.

Kinetic data have previously been obtained for ChiAwt, ChiBwt, ChiB-D140N, and ChiB-D142N (e.g., refs 37 and 49−51) though most of these studies were based on the use of chromogenic or fluorogenic substrates, and only for ChiAwt and ChiBwt[49,51] have natural substrates been used. We have in this work determined k_{cat} and K_m using the natural substrate $(GlcNAc)_4$ (Table 1). In the substrate concentration range studied, all of the mutants showed straightforward Michaelis−Menten kinetics without substrate inhibition, as also previously shown for the wild-type enzymes[49] (Figure 3 and Figures S7 and S13). At the concentrations used, no transglycosylation was observed within the time frame of the assay. Furthermore, initial rates when $(GlcNAc)_6$ (Table S2) and $(GlcNAc)_3$ (Table S3) were substrates were also determined.

In general, it is observed that mutations of aspartates in ChiA yielded activities that are below 0.1% of the wild-type activity except Asp to Asn in position 313 (ChiA-D313N) where as much as 2% activity is retained. There is no clear trend in observed K_m values for the single mutations. Asp to Ala exchange in position 311 yielded equal value as for the wild-type while the Asp to Asn gave a 4-fold increase. In position 313, the Asp to Ala exchange yielded a 9-fold increase while a ∼3-fold decrease was observed for the Asp to Asn. For ChiB, mutations of the same aspartates followed the same trend as observed for ChiA with respect to

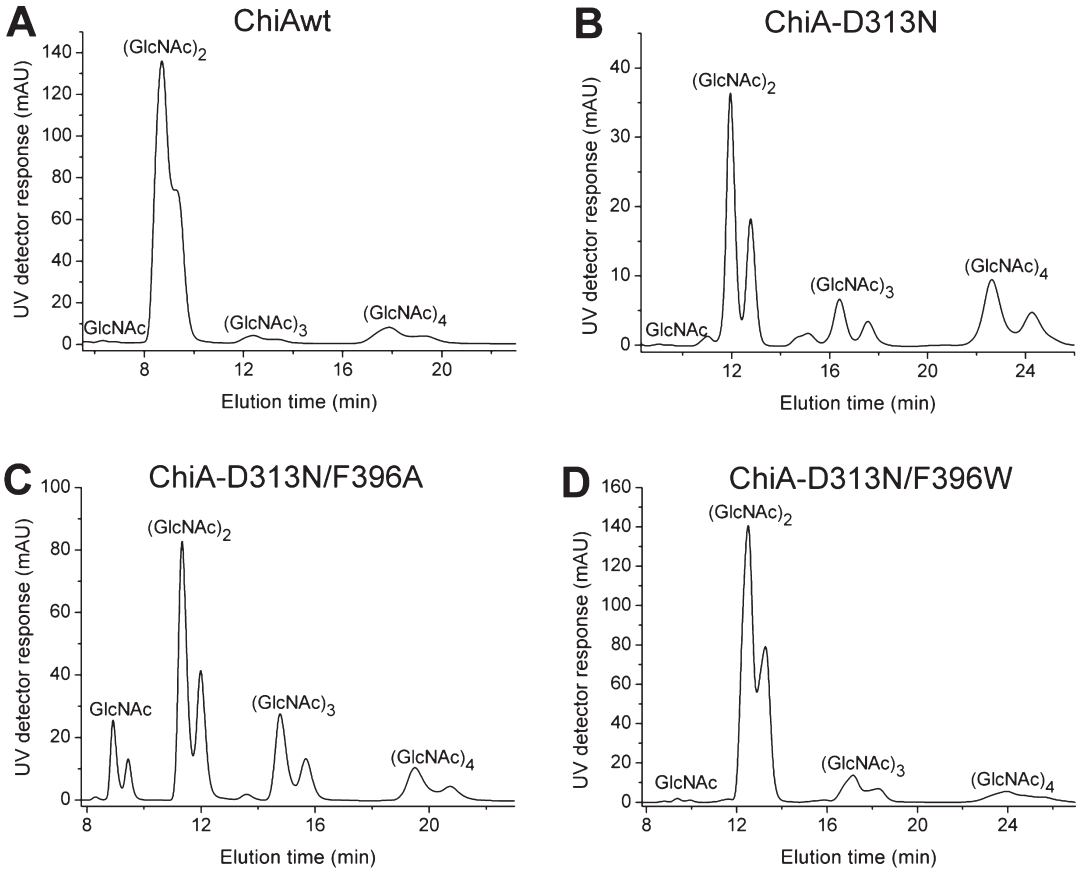

Figure 6. HPLC chromatograms illustrating transglycosylation activity. Reactions were run under "transglycosylation conditions" by incubating 1.0 mM $(GlcNAc)_4$ with (A) ChiAwt for 20 s, (B) ChiA-D313N for 120 min, (C) ChiA-D313N/F396A for 40 min, or (D) ChiA-D313N/F396W for 40 min (all at concentration of 3.0 μM) at 37.0 °C in 20 mM sodium acetate (0.1 mg/mL BSA, pH 6.1). The chromatograms are primarily meant to illustrate variation in the $(GlcNAc)_3$/GlcNAc ratio (close to 1 for ChiAwt and ChiA-F396A; >1 for the most transglycosylating mutants). Note that the oligomer peaks are split in two, with the α-anomer eluting immediately before the β-anomer.

activities. There was though an increase in all K_m values except for ChiB-D142N that, as observed for the analogue ChiA-D313N, yielded a decrease (4-fold).

Interestingly, the Trp to Ala exchange in the +1 subsite in the ChiB-D142N mutant resulted in a ~4-fold increase in activity while the Phe exchange to either Trp or Ala in the +2 subsite in the analogue ChiA-D313N gave 4- and 25-fold reduction, respectively. As expected, the exchange of an aromatic amino acid with an Ala yielded an increase in observed K_m values. Even so, the increase in the +2 subsite for ChiA was only 2-fold, while it was 19-fold for ChiB in the +1 subsite. Another interesting observation is that the Phe to Trp exchange in for ChiA in the +2 subsite only yielded a slight decrease in K_m (equal values within experimental errors).

Detecting Transglycosylation Using Mass Spectrometry. As a first qualitative attempt, $(GlcNAc)_4$ was incubated with ChiAwt, ChiA-D313N, ChiA-D313N/F396W, and ChiA-D313N/F396A, chitinases with mutations that were expected to have effect with respect to transglycosylation activity (described above), under transglycosylating conditions (high enzyme and substrate concentrations).[19,52] MALDI-TOF-MS was used to see if longer products in the early phases of the reaction could be detected with the results depicted in Figure 4. While no products longer than $(GlcNAc)_4$ were detected for the wild-type enzyme, $(GlcNAc)_5$ and $(GlcNAc)_6$ were detected for several of the mutants. The product spectrum for the most transglycosylating variant in this study (ChiA-D313N/F396W) also showed longer oligomeric products (Figure 4E).

Relative Quantification of Transglycosylation Using Natural Substrates. Transglycosylation activities of chitinases have previously been monitored by both using artificial[21] and natural substrates.[19,23] A clear disadvantage in using artificial substrates is that the chromophores (4-methylumbelliferyl or *p*-nitrophenol) interact differently with chitinases, i.e., ChiB, compared to a GlcNAc moiety.[49] A difficulty in using natural substrates is that the transglycosylation products are intermediates in consequent hydrolytic and transglycosylating events. Even so, the use of natural substrates compared to artificial is clearly beneficial when determining what structural features within the enzyme that affect transglycoysylation activity. For the chitinases investigated in this work, the use of the natural substrate $(GlcNAc)_4$ allows for a relative quantification of transglycosylation. Interaction of a $(GlcNAc)_4$ molecule with the active site of the investigated chitinases is shown in Figure 5. A productive binding yields a dimeric oxazolinium ion in subsites −2 and −1. A transglycosylation reaction can take place when a new $(GlcNAc)_4$ (most abundant sugar acceptor initially) combines with the oxazolinium ion to give a $(GlcNAc)_6$ molecule. $(GlcNAc)_6$ can bind productively from −4 to +2 (major) and −3 to +3 (minor) (Figure 5 and Table S2). The minor binding mode (−3 to +3) produces only $(GlcNAc)_3$ that is a very poor substrate with a 40-

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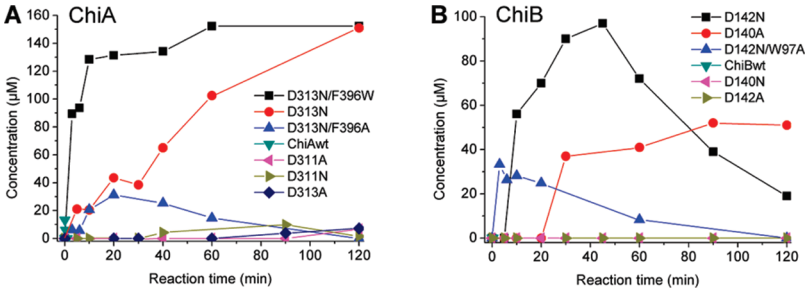

Figure 7. Transglycosylation by mutants of ChiA and ChiB with $(GlcNAc)_4$ as substrate. The plots show the concentrations of $(GlcNAc)_3$ formed during the reaction with 3.0 μM of (A) a ChiA variant or (B) a ChiB variant with 1.0 mM $(GlcNAc)_4$ in 20 mM ammonium acetate (0.1 mg/mL BSA, pH 6.1) at 37 °C. Information about the concentrations of other oligomers over time (i.e., monomers, dimers, and tetramers) is provided in Figures S14 and S15 of the Supporting Information for ChiA and ChiB variants, respectively. Since under these conditions ChiA converts some $(GlcNAc)_4$ directly to $(GlcNAc)_3$ and GlcNAc, trimer concentrations were corrected for the amount of observed monomers, i.e., for the amount of trimers that are not a result of transglycosylation. See text for more details.

and 30-fold lower initial activity compared to $(GlcNAc)_6$ and $(GlcNAc)_4$, respectively (Table 1 and Tables S2 and S3). Furthermore, $(GlcNAc)_3$ binds more than 3 and 2 orders of magnitude weaker to ChiB than $(GlcNAc)_6$ and $(GlcNAc)_4$, respectively.[42] Because of this, $(GlcNAc)_3$ accumulates and can be quantified using HPLC and directly related to the extent of transglycosylation. This can be done since the direct conversion of $(GlcNAc)_4$ to $(GlcNAc)_3$ and GlcNAc cannot be detected for ChiB and happens at very low frequency for ChiA (Table S4) and can be corrected for, and productive binding of $(GlcNAc)_6$ is similar for the most transglycosylating mutants (percentage of −3 to +3 binding mode for $(GlcNAc)_6$ between 11 and 16%, Table S2).

Even though $(GlcNAc)_3$ is a substrate, it is important to emphasize that its use only results in an underestimation of the transglycosylation activity and that this disadvantage is minute compared to the perturbation posed by artificial substrates.

Using this approach, patterns of shorter oligomeric products (Figure 6 and Figures S14 and S15 of the Supporting Information) indicated that several of the mutants are able to transglycosylate. The ChiA-D313N, ChiA-D313N/F396A, and the ChiA-D313N/F396W mutants produced considerable amounts of $(GlcNAc)_3$ at a much higher concentration than GlcNAc (Figures 6 and 7A), indicative of transglycosylation in accordance with the mass spectrometry analysis (Figure 4). Three of the ChiB mutants, ChiB-D140A, ChiB-D142N, and ChiB-D142N/W97A, also showed signs of transglycosylation activity (Figure 7B). ChiA mutants ChiA-D311N, ChiA-D311A, and ChiA-D313A produced mainly $(GlcNAc)_2$, and very small, equal amounts of GlcNAc and $(GlcNAc)_3$ and ChiB mutants ChiB-D140N and ChiB-D142A converted $(GlcNAc)_4$ exclusively to two molecules of $(GlcNAc)_2$, suggesting normal hydrolytic activities. ChiBwt showed no transglycosylation activity in the reaction with $(GlcNAc)_4$, while ChiAwt produced a small "excess" of $(GlcNAc)_3$ (excess relative to GlcNAc). The extent of transglycosylation activities are listed in Table 1.

DISCUSSION

Kinetic Characterizations. Mutations in the DxDxE motif yielded enzymes with greatly reduced activities against $(GlcNAc)_4$ for both ChiA and ChiB. A recent study using quantum mechanics/molecular mechanics (QM/MM)[53] demonstrated the importance of the Asp^{142} residue in the catalysis. The reaction mechanisms of both the wild-type ChiB from *S. marcescens* and the ChiB-D142N mutant were investigated by modeling the first step in the reaction with a GlcNAc substrate (glycosylation step, see Figure 1B,C). It was shown that both enzymes follow the same reaction mechanism but that the ChiB-D142N mutant stabilizes the transition state and the oxazolinium ion intermediate to a lesser extent than the wild-type enzyme. The $Asp^{311/140}$ mutations could in principle have similar, perhaps more indirect, effects, since these Asp residues are directly coupled to the "up and down movement" of $Asp^{313/142}$ during catalysis (Figure 1).[32] Obviously, the inserted alanine residues in $Asp^{313/142}$ have few interactions with the substrate and by this will stabilize the transition state even less.

The increase in k_{cat} for ChiB and the decrease observed for ChiA upon removal of aromatic residues in +1 and +2 subsite, respectively, may be a reflection of that ChiA and ChiB degrade chitin in opposite directions.[40,54] Positive subsites are where product is released in ChiA while for ChiB it is where the polymeric substrate remains attached during processive hydrolysis.[40,54,55] Binding strength measurement of allosamidin to negative subsites (polymeric substrate binding site in ChiA and product release site for ChiB) revealed that even though binding was equally strong there were huge differences in the thermodynamic signatures.[38] Binding was driven by enthalpic changes (-6.2 ± 0.2 kcal/mol) for ChiA in accordance with this being substrate binding subsites while it was endothermic for ChiB (3.8 ± 0.2 kcal/mol). It appears that the positive subsites in ChiA and negative subsites in ChiB not only bind substrate but also are parts of the product displacement mechanism.

Origin of Transglycosylation. Transglycosylation is a common phenomenon in glycoside hydrolases and can in principle be controlled by favoring binding and correct positioning of a sugar to the intermediate (see Figure 5, step 2b). Affinity for sugar acceptors may to some extent be reflected in the Michaelis–Menten constant K_m. Correlations between a lower K_m and increased transglycosylation have been observed for β-mannanase Man5A from blue mussel *Mytilus edulis*[56] and for endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis*.[16] It is clear from our data that removal of aromatic residues (ChiA-D313N/F396A and ChiB-D142N/W97A), and by this increasing the K_m value, decreases the extent of transglycosylation. Most interestingly, exchanging Phe to Trp (ChiA-D313N/F396W) only yielded a slight decrease in the K_m value (equal within experimental errors) but produced the same high level of $(GlcNAc)_3$ as the ChiA-D313N mutant, but at a much faster rate (Figure 7A). It is

conceivable that even though the stacking of a GlcNAc moiety with Phe and Trp may be of equal strength, the larger aromatic surface area of Trp compared to Phe may attract an incoming sugar acceptor faster and also improve the positioning of the sugar acceptor and by this be the origin of the quicker rate in transglycosylation.

Even though the single mutants that showed the highest transglycosylation activities, ChiA-D313N and ChiB-D142N, also showed 2−3-fold decreased K_m values, compared to the wild-type enzymes (Table 1), other factors such as positioning and activation of the catalytic water and optimal aligning of the acceptor are likely to play a role. Crystallographic studies of ChiB[30] have shown that the D142N mutation leads to subtle changes in the apoenzyme, where catalytically important residues adopt conformations that are normally seen in the enzyme−substrate complex. There are no similar structural data for ChiA-D313N, but taking into account the high structural similarity between the catalytic domains of ChiA and ChiB (Figure 2 and refs 33, 36, 57, and 58) as well as the similar effects of the $Asp^{313/142} \rightarrow$ Asn mutations on hydrolytic properties (Table 1), it seems safe to assume the role of $Asp^{313/142}$ and the effects of mutating this residue are similar in both enzymes. $Asp^{313/142}$ has multiple roles in catalysis in family 18 chitinases (Figure 1; e.g. ref 32), where it (1) affects the positioning and nucleophilicity of the acetamido group involved in catalysis, (2) interacts with the catalytic acid, presumably contributing to the necessary cycling of its pK_a during catalysis and thus to the positioning and/or activation of the catalytic water, and (3) interacts with and stabilizes the oxazolinium ion intermediate. Both the ChiA and the ChiB data show that mutation of $Asp^{313/142}$ to Asn has profound effects on transglycosylation activity. In the study using QM/MM discussed above, it was observed that for ChiBwt the boat conformation of the intermediate is most stable, whereas for ChiB-D142N the chair conformation is most stable. Whether this effect contributes to the transglycosylation activity of the ChiB-D142N mutant is not known, but clearly, this mutation leads to a change in active site electrostatics that could lead in some way to lower hydrolyzability of or increase the probability of nucleophilic attack by a sugar on the oxazolinium ion intermediate, perhaps due to effects on the catalytic water.

It is important to note that there is no correlation between the k_{cat} for hydrolysis and apparent transglycosylating activity neither; one might expect that a low k_{cat} could help in attaining high apparent transglycosylation levels, but in the present case the most transglycosylating mutants are among those with the highest k_{cat} (Table 1).

The dramatic increase in substrate conversion rate in ChiA-D313N/F396W suggests that the deglycosylation step (Figure 5, steps 2a and 2b) is the rate-limiting step in catalysis by ChiA-D313N. Upon adding F396W, deglycosylation is enhanced by faster transglycosylation (Figure 7), rather than by faster hydrolysis (Table 1), hence creating a hypertransglycosylating mutant.

In conclusion, our results show how the transglycosylation activity of glycoside hydrolases may be manipulated and improved. Because of an increased demand for bioactive carbohydrate oligomers, it is pertinent to have access to several technologies yielding oligomeric products of desired isomerism in high yields. An example is inhibition of the human acidic mammalian Chitinase (AMCase), a family 18 Chitinase that is a therapeutic target in asthma amelioration.[4] Chitooligosaccharides with specific number and sequences of acetylated and deacetylated units have been shown to inhibit family 18 chitinases due to preferences of acetylated sugar moieties in individual subsite yielding nonproductive binding.[59] Relatively short chitooligosaccharides are easily produced by enzymatic hydrolysis of chitosan,[59] a partially, deacetylated soluble form of chitin, and can by transglycosylation technology be used to produce longer oligosaccharides of specific length and sequence of acetylated and deacetylated units. Partially deacetylated chitooligosaccharides are hydrolyzed slower than fully acetylated, which is beneficial when using transglycosylases. Moreover, transglycosylation technology is complementary to glycosynthase technology, which requires the use of mutated glycosyl hydrolases that are no longer hydrolytically active,[60,61] where the need for modified glycosyl donors is a disadvantage. Future research should focus on constructing mutants that have even lower hydrolytic activity and similar or increased transglycosylation activities as observed in this work.

■ ASSOCIATED CONTENT

Supporting Information. Table S1: oligonucleotide primers used for site-directed mutagenesis; Table S2: specific activities toward $(GlcNAc)_6$ and fraction of $(GlcNAc)_6$ converted to two trimers; Table S3: specific activity toward $(GlcNAc)_3$; Table S4: amount of $(GlcNAc)_3$ that is formed in the kinetic experiments with $(GlcNAc)_4$ substrate; Figures S1−S13: data used for Michaelis−Menten analysis and the Michaelis−Menten plots; Figures S14 and S15: product profiles of the degradation of $(GlcNAc)_4$. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

BSA, bovine serum albumin; ChiA, Chitinase A from *Serratia marcescens*; ChiB, Chitinase B from *Serratia marcescens*; GlcN, glucosamine; GlcNAc, N-acetylated glucosamine; HPLC, high-pressure liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PDB, protein data bank; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; QM/MM, quantum mechanics/molecular mechanics; wt, wild-type; 4-MU-$(GlcNAc)_2$, 4-methylumbelliferyl *N,N′* diacetylchitobiose.

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