ACS chemical biology

pubs.acs.org/acschemicalbiology

Articles

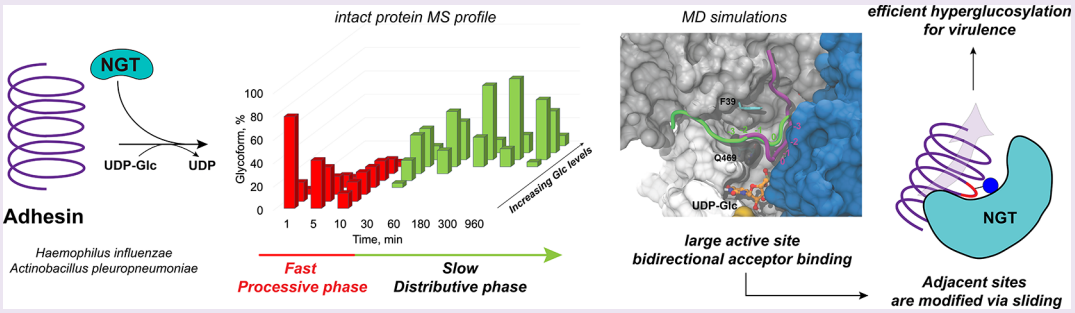
Semiprocessive Hyperglycosylation of Adhesin by Bacterial Protein *N*-Glycosyltransferases

Liubov Yakovlieva, Carlos Ramírez-Palacios, Siewert J. Marrink, and Marthe T. C. Walvoort*

Cite This: *ACS Chem. Biol.* 2021, 16, 165−175

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information



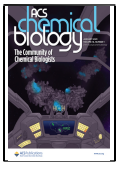
ABSTRACT: Processivity is an important feature of enzyme families such as DNA polymerases, polysaccharide synthases, and protein kinases, to ensure high fidelity in biopolymer synthesis and modification. Here, we reveal processive character in the family of cytoplasmic protein *N*-glycosyltransferases (NGTs). Through various activity assays, intact protein mass spectrometry, and proteomics analysis, we established that NGTs from nontypeable *Haemophilus influenzae* and *Actinobacillus pleuropneumoniae* modify an adhesin protein fragment in a semiprocessive manner. Molecular modeling studies suggest that the processivity arises from the shallow substrate binding groove in NGT, which promotes the sliding of the adhesin over the surface to allow further glycosylations without temporary dissociation. We hypothesize that the processive character of these bacterial protein glycosyltransferases is the mechanism to ensure multisite glycosylation of adhesins *in vivo*, thereby creating the densely glycosylated proteins necessary for bacterial self-aggregation and adherence to human cells, as a first step toward infection.

INTRODUCTION

Protein glycosylation is a ubiquitous post-translation modification wherein amino acid side chains of proteins are decorated with carbohydrates. Glycosylation affects many properties of the modified protein (e.g., solubility, stability, transport) and influences the biochemical pathways that the glycoprotein is involved in, such as signaling, communication, and interaction with receptors.[1] Interestingly, the majority of glycoproteins feature complex glycans attached at specific positions (e.g., antibodies), and their truncation or absence can greatly influence the function of the glycoprotein and the downstream processes (e.g., in cancer).[2] On the other hand, there are examples of glycoproteins where the sheer number of carbohydrate modifications seems to be more important for biological activity than the specific location. For instance, in the case of mucins, several *O*-GalNAc-transferases, each with specific substrate specificity, work in concert to create a densely covered glycan surface.[3] In bacteria, an increasing number of proteins are known to be densely glycosylated (hyperglycosylated), and these proteins are often involved in virulence traits such as adhesion and autoaggregation.[4]

Little is known about the mechanistic aspects of protein hyperglycosylation (or multisite glycosylation) and how protein glycosyltransferases (GTs) control the efficiency of surface modification. The majority of the biosynthetic processes that produce glycoproteins can broadly be divided into two categories, i.e., enzymes involved in *N*-glycosylation that transfer a preassembled lipid-linked glycan *en bloc* to an asparagine residue in the consensus sequence N-X-(S/T) (where X ≠ Pro), such as the well-known eukaryotic OST complex[5] and its bacterial homologue PglB,[6] and enzymes responsible for *O*-linked glycosylation, that transfer single carbohydrate residues from soluble nucleotide-activated substrates to serine and threonine, such as *O*-GlcNAc transferase (OGT)[7] and *O*-GalNAc transferases involved in the initiation of mucin glycosylation.[3] *N*-linked glycosylation occurs predominantly cotranslationally on a limited number of residues, and subsequent trimming and/or further modification of the glycan results in a tremendous diversity in glycoforms, as exemplified by the >200 erythropoietin glycoforms identified

Received: October 30, 2020
Accepted: December 22, 2020
Published: January 6, 2021



https://dx.doi.org/10.1021/acschembio.0c00848
ACS Chem. Biol. 2021, 16, 165−175

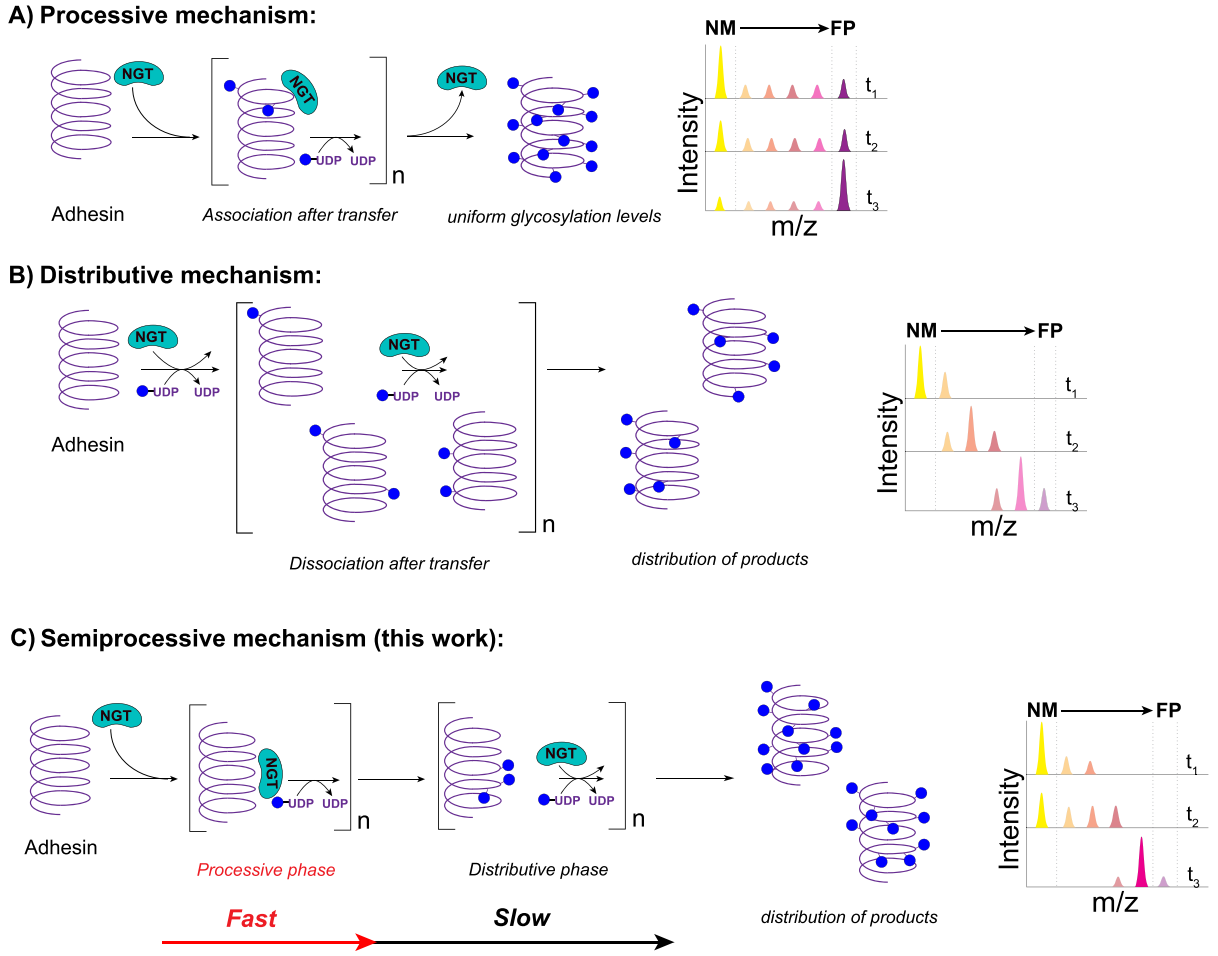


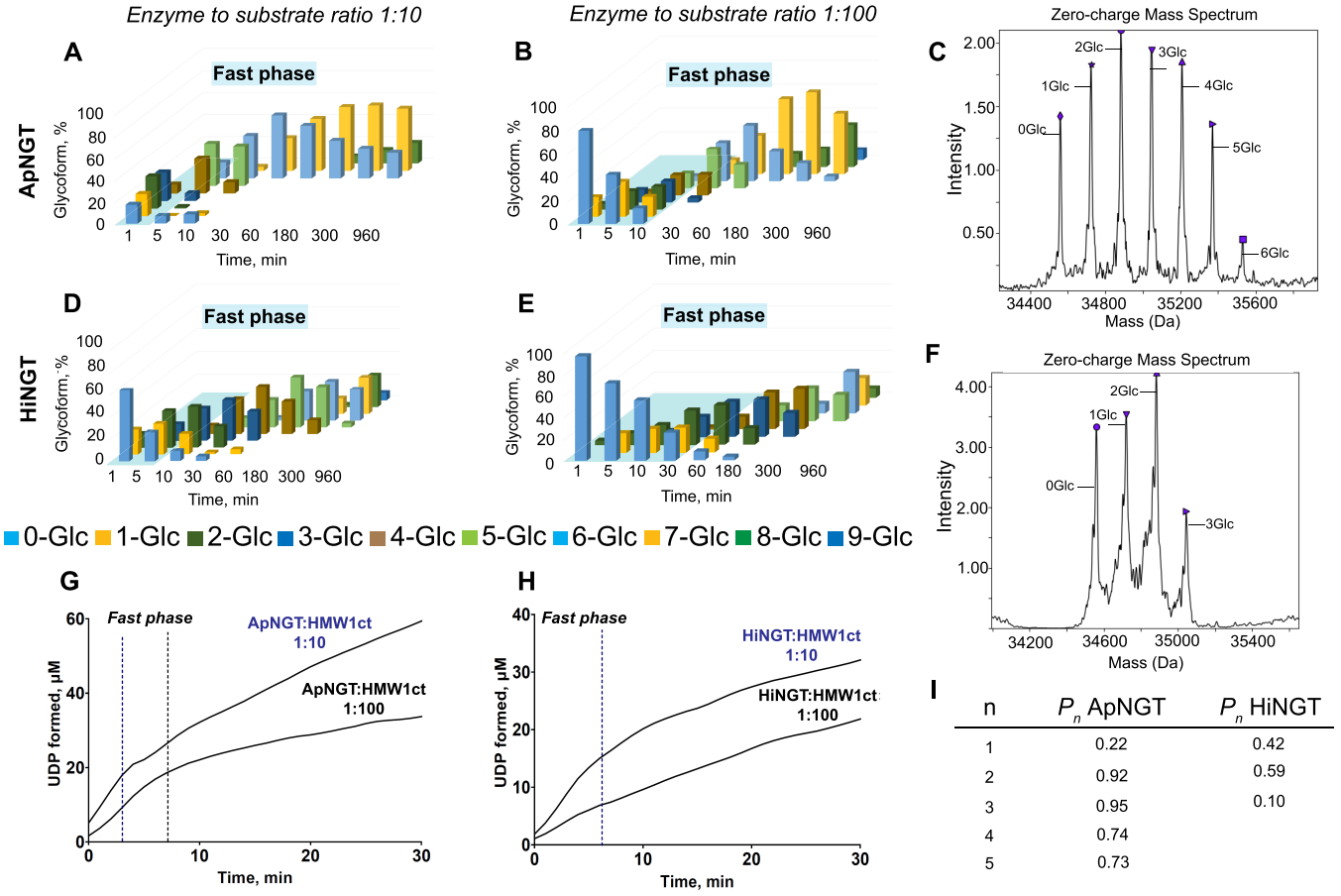
Figure 1. Schematic representation of the mechanism and product profiles in (A) a processive mechanism, (B) a distributive mechanism, and (C) the semiprocessive mechanism of adhesin hyperglycosylation proposed in this work. Individual peaks in the MS spectrum illustration represent the addition of the single glucose. Transparent peaks represent intermediate glycoforms. NGT = *N*-glycosyltransferase, NM = nonmodified substrate, FP = final product, blue circle = glucose.

in a single sample.[8] On the other hand, *O*-linked glycosylation mostly happens post-translationally and is often driven by nucleotide-sugar substrate concentrations.[9]

An intriguing glycosylation system that combines characteristics of both categories is the family of cytoplasmic *N*-glycosyltransferases (NGT), which is unique to bacteria. The first NGT, called HMW1C, was identified in nontypeable *Haemophilus influenzae* (NTHi)[10,11] and is responsible for the multisite glycosylation of high-molecular weight (HMW) adhesin HMW1A. Together with the translocator HMW1B, this two-partner secretion system produces densely glycosylated adhesins on the extracellular surface of NTHi, which are crucial for adherence to human epithelial cells, as the first step in infection. Soon after this first report, homologous NGTs were identified in *Actinobacillus pleuropneumoniae*,[12] *Yersinia enterocolitica*,[13] *Kingella kingae*, and *Aggregatibacter aphrophilus*.[14] NGTs generally catalyze the transfer of a single glucose (Glc) residue from the nucleotide-activated donor UDP-α-D-Glc to an asparagine residue in the consensus sequence (N-X-S/T). They are metal-independent inverting GTs, creating a β-linked modification, and based on structural similarities are classified in GT family 41 (CAZy database),[15,16] together with the soluble *O*-GlcNAc transferase (OGT) as the only other member. Interestingly, NGTs display a relaxed sequence requirement, as modification on nonsequon Asn residues, and modification on residues other than Asn have been observed.[17] Moreover, also dihexose modifications have been identified both *in vivo* and *in vitro*, suggesting that NGTs may have the ability to generate both protein *N*-linkages and glycan *O*-linkages.[10,18] The majority of known acceptor substrates of NGTs belong to the class of adhesins and autotransporters, which are generally large membrane-associated proteins that play a distinct role in virulence.[19,20] It is noteworthy that in almost all examples where *N*-linked glucosylation activity was confirmed, a large number of glucose moieties was added to the native protein substrates.[17,18] The importance of multisite glycosylation for adherence was confirmed when heterologous coexpression of KkNGT and its autotransporter substrate Knh in a nonadherent *E. coli* resulted in bacterial adherence to human epithelial cells.[14]

To unravel the mechanism of bacterial multisite protein glycosylation, we questioned whether hyperglycosylation is the result of a processive mechanism in NGT. This research question was inspired by the fast modification by ApNGT of the *C*-terminal fragment of HMW1A adhesin that we observed when producing *in vitro* glucosylated adhesin fragments for antibody binding studies.[21] Processivity is a complex mechanistic feature that has been identified in a variety of enzymes, including DNA polymerases, ubiquitin ligases, protein kinases, and enzymes involved in polysaccharide

https://dx.doi.org/10.1021/acschembio.0c00848
ACS Chem. Biol. 2021, 16, 165−175



n	P_n ApNGT	P_n HiNGT
1	0.22	0.42
2	0.92	0.59
3	0.95	0.10
4	0.74	
5	0.73	

Figure 2. Time-course experiments and kinetic parameters of the glycosylation reaction of HMW1ct with ApNGT and HiNGT. (A) Time-course product profile of ApNGT and HMW1ct in a ratio of 1:10. (B) Time-course product profile of ApNGT and HMW1ct in a ratio of 1:100. (C) Deconvolved mass spectrum of the product profile generated from 1:100 ApNGT/HMW1ct at 10 min. (D) Time-course product profile of HiNGT and HMW1ct in a ratio of 1:10. (E) Time-course product profile of HiNGT and HMW1ct in a ratio of 1:100. (F) Deconvolved mass spectrum of the product profile generated from 1:10 HiNGT/HMW1ct at 5 min. For panels A–F, every reaction contained 10 μM of HMW1ct protein substrate, and the molarity of the enzyme was adjusted according to the desired ratio. UDP-Glc is present in excess (1 mM). Representative data of two independent experiments are shown. Deconvolved spectra for selected time points are available in Supplementary Figures S2–S5. The light blue panel highlights the processive fast phase. (G) Reaction progress continuously monitored with the coupled-assay for ApNGT. (H) Reaction progress continuously monitored with the coupled-assay for HiNGT. (I) Processivity parameters obtained for ApNGT and HiNGT.

synthesis and breakdown (glycosyl transferases and hydrolases)[22] but has not yet been identified in protein GTs. In a processive mechanism, NGT would modify the adhesin substrate with multiple glucoses during a single substrate binding event (Figure 1). Because multiple rounds of catalysis happen before dissociation, a processive mechanism would result in the fast generation of multiply glycosylated proteins. Alternatively, NGT may employ a distributive mechanism, in which every binding event is followed by glucose transfer and release of the resulting product (Figure 1B). For a subsequent modification, the adhesin substrate has to bind again, and as a result, modifications would be introduced in a stepwise manner and products reflect a distribution of modifications. A distributive mechanism has been observed for the OGT-catalyzed O-GlcNAcylation of RNA polymerase II.[23] Processivity is a challenging trait to study, and established methods have been reviewed elsewhere.[22,24]

We selected HiNGT (R2846_0712) and its close homologue ApNGT (APL_1635, 65% identity and 85% similarity),[12] and using the C-terminal region of the natural HMW1A adhesin (HMW1ct, from *H. influenzae*, Figure S1) as an acceptor substrate, we show that both NGTs display semiprocessive behavior (Figure 1C). Moreover, using molecular dynamics simulations we provide insight into the structural factors that may be at the basis of adhesin hyperglycosylation. Our research establishes a novel mechanism in the family of protein *N*-glycosyltransferases that will advance our understanding of bacterial protein hyperglycosylation and is important for the application of the NGT system in glycoprotein production.

RESULTS

Glycosylation of HMW1ct Proceeds *via* an Initial Fast Processive Phase. To get a first impression of the glycosylation efficiency on the adhesin substrate HMW1ct, the reaction by ApNGT and HiNGT was monitored over time by examining the product profiles. *In vitro* reactions were performed at RT with varying enzyme to substrate ratios (UDP-Glc was always present in large excess) and quenched at certain time points by heating to 100 °C for 10 min. Reaction aliquots were then subjected to intact protein LC-MS analysis, and conversion was calculated from the ion intensities of the nonmodified substrate and glycoforms observed in the MS spectra. Ionization differences between the different glyco-

https://dx.doi.org/10.1021/acschembio.0c00848
ACS Chem. Biol. 2021, 16, 165–175

forms were not significant enough to introduce a correction factor.

As depicted in Figure 2A when the ratio ApNGT to HMW1ct adhesin was 1:10 (molar ratio), glycosylation occurred rapidly and led to the formation of a mixture of 3−6 times glucosylated (3-Glc to 6-Glc) product within 5 min. Over the next 15 h, this 6-Glc product was slowly but steadily converted to even higher-order glycoforms (7-Glc and 8-Glc). Interestingly, in the first minute of the reaction, no significant accumulation of a single early glycoform was observed but rather a broad distribution of 1-Glc to 4-Glc products. Moreover, low levels of the substrate and early glycoforms (0-Glc to 2-Glc) persisted in the first 10 min. To slow down the rate of product formation and capitalize on intrinsic binding affinity instead of concentration effects, the experiment was repeated with a ratio of ApNGT to HMW1ct adhesin of 1:100 (Figure 2B). The product profile thus obtained provided a more pronounced effect, in which early and intermediate glycoforms are rapidly produced, resulting in low level accumulation of intermediate products (1-Glc to 6-Glc) in 10 min (Figure 2C), which are subsequently converted to 7-Glc and 8-Glc as the major products after 15 h. The absence of significant levels of one intermediate glycoform before 30 min is intriguing, as is the persistence of nonmodified substrate (0-Glc) while advanced glycoforms are being produced. While the adhesin substrate is present in large excess (enzyme/substrate is 1:100), especially at the beginning of the reaction, it appears that, for ApNGT, formation of the first glycoform triggers the production of the next one in a processive manner. Using a continuous assay that quantifies UDP release, a clear transition from the fast phase to the slow phase was also observed (Figure 2G). Close inspection of the progress curve of 1:100 ApNGT/HMW1ct reveals a short "lag-phase" in the first minutes, where the rate of UDP formation quickly increases, indicative of the increasing affinity of ApNGT for the early glycoform products. In an attempt to quantify this early processive behavior, the processivity factor P_n was calculated using the profile at 10 min (Figure 2I, Table S1). The P_n value reflects the probability that the enzyme will remain associated with the modified substrate to add an additional modification ($n + 1$) instead of dissociating.[25,26] The P_n value for the first addition was 0.22, which suggests that only 22% of ApNGT that added the first glucose continued on to add more modifications. Intriguingly, the P_n values for the next two additions were high (0.92 and 0.95, respectively), revealing that the production of the 3-Glc and 4-Glc products happens with considerable processivity. Importantly, the change from low to high P_n values between the first and second Glc additions may reflect a "priming" step, i.e., formation of the preferred partially glycosylated substrate. Subsequently, the P_n value drops to 0.74 (for 5-Glc) and 0.34 (for 6-Glc), which supports a change to a more distributive mechanism.

The HiNGT-catalyzed HMW1ct glycosylation appears to produce product profiles that share characteristics with the profiles from ApNGT; however the trend is less pronounced and develops at a significantly slower rate. When the reaction was performed with a ratio of HiNGT to adhesin of 1:10 (Figure 2D), a broad distribution of glycoforms (1-Glc to 3-Glc) was formed in the first 5 min (Figure 2F). Subsequently, these glycoforms were gradually further modified to reach mixtures where the major products were 2-Glc and 3-Glc (10 min), 3-Glc and 4-Glc (30 min), 4-Glc and 5-Glc (90 min), and 5-Glc and 6-Glc (300 min). After 15 h, the final glycoforms contained mostly 7−9 Glc moieties. This period in which a batch of glycoforms is collectively modified to produce more substituted products yields a product profile that resembles a Poisson distribution,[27] which is associated with a distributive mechanism. Performing the reaction with a ratio of HiNGT to adhesin of 1:100 (Figure 2E) again emphasized the processive behavior in the first phase, where early glycoforms are rapidly generated while the nonmodified substrate (0-Glc) persists for at least 180 min. Progress curves obtained with the continuous coupled-assay again indicate a change from a fast phase to a slow phase, especially for a ratio of 1:10 HiNGT/HMW1ct (Figure 2H). In the case of HiNGT, the P_n parameters (at 30 min, Figure 2I, Table S2) for the first additions were 0.42 (to 2-Glc), 0.59 (to 3-Glc), and 0.10 (to 4-Glc), suggesting that most processive character was displayed at the addition of the third glucose.

To quantify the difference in reaction kinetics between ApNGT and the slower HiNGT, we determined k_{cat} and K_m using the continuous coupled-assay (Figure S6). ApNGT followed typical Michaelis−Menten kinetics, which has been linked to processive character in the case of multisite phosphorylation, resulting in k_{cat} = 0.74−0.99 s^{-1} and K_m = 6.09−15.6 μM.[28,29] In contrast, for HiNGT, the initial velocities (V_0) were found to increase linearly and did not reach a maximum level at the highest HMW1ct concentration (Figure S7). This suggests that the activity of HiNGT is more dependent on the HMW1ct concentration than is the case for ApNGT. In addition, we postulate that especially in the case of HiNGT, higher HMW1ct concentrations lead to a fast production of inhibitory products (*vide infra*). In analogy to studies on multisite phosphorylation,[30] this product inhibition may stem from a more distributive character. These experiments together paint a picture in which ApNGT, in particular, displays processive behavior in the initial fast phase, followed by a transition to a slower phase with more distributive characteristics. HiNGT seems to follow the same trend, albeit with a shorter fast processive phase.

Product Inhibition Causes a Mechanism Change and Determines the Final Product Profile. With the production of 5-Glc and 6-Glc for ApNGT (30 min, Figure 2B) and 2-Glc and 3-Glc for HiNGT (60 min, Figure 2E), the reaction seems to enter into a slow phase that has a more distributive character. Because it was observed previously that ApNGT has a high affinity for the Glc-adhesin product, which seriously hampered the purification by standard methods,[17,21] we hypothesized that this mechanistic transition was due to a competing binding of the glycosylated products. The affinity of ApNGT toward substrate (HMW1ct) or product (Glc-HMW1ct, mixture of 7,8,9,10-Glc glycoforms) was determined using surface plasmon resonance (SPR). Interestingly, the K_D values were in the same range (HMW1ct K_D = 5.85 ± 4.49 μM, Glc-HMW1ct K_D = 9.81 ± 1.55 μM), suggesting that ApNGT binds both the substrate and the product with equal affinity (Figure S8). Unfortunately, we were not able to perform the same studies with HiNGT, as concentrated solutions of the enzyme were not stable enough for SPR experiments.

On the basis of the similar affinities of ApNGT for both the adhesin substrate (HMW1ct) and product (Glc-HMW1ct), we set out to evaluate the influence of concentration on the extent of glycosylation. We hypothesized that if the production of glycosylated product interferes with the efficiency of the reaction, increasing the substrate concentration will enhance

https://dx.doi.org/10.1021/acschembio.0c00848
ACS Chem. Biol. 2021, 16, 165−175

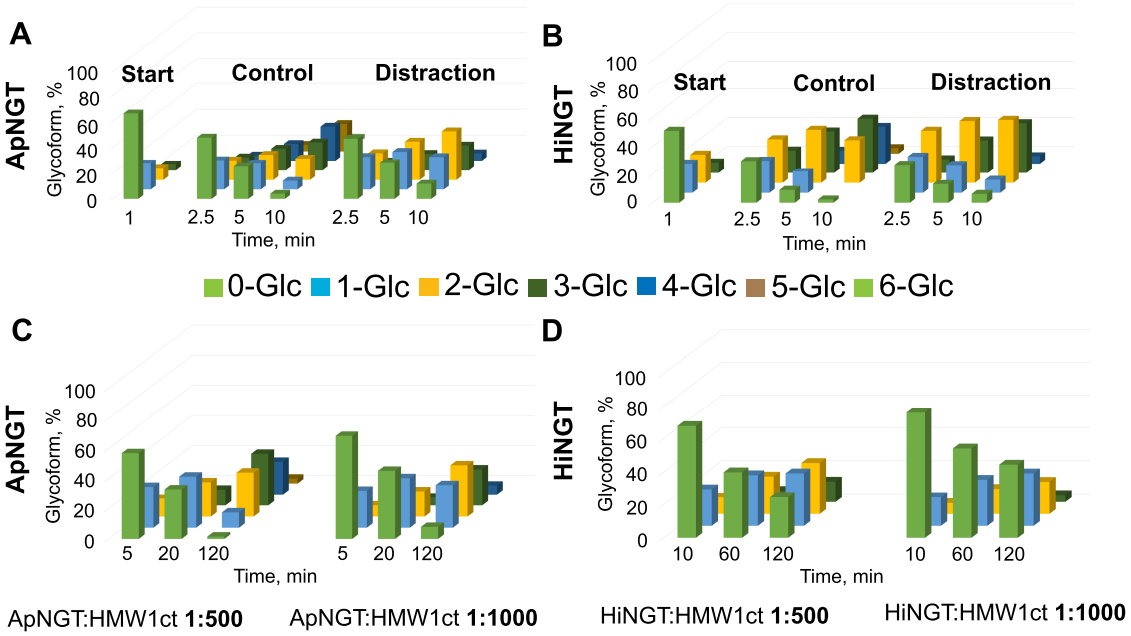


Figure 3. Distraction and single-hit experiments. (A) ApNGT/HMW1ct (1:100) was reacted for 1 min, followed by the addition of additional 10 μM Glc-HMW1ct. (B) HiNGT/HMW1ct (1:10) was reacted for 1 min, followed by the addition of additional 10 μM Glc-HMW1ct. (C) Time-course experiments with ApNGT/HMW1ct at a ratio of 1:500 and 1:1000. (D) Time-course experiments with HiNGT/HMW1ct at a ratio of 1:500 and 1:1000. Representative data of two independent experiments are shown. Deconvolved mass spectra for selected time-points are available in Supplementary Figure S11.

the production of these inhibitory glycoforms, resulting in an overall reduced glycosylation efficiency. This effect has been observed before in an *ex vivo* expression system of HiNGT and HMW1A (full-length *H. influenzae* adhesin), where the increasing expression of HMW1A resulted in a reduction of site-specific glycan occupancy.[31] We performed overnight glycosylation reactions in which the ratio of ApNGT/HMW1ct was kept constant at 1:100, and the ratio of HiNGT/HMW1ct at 1:10, while the concentration of HMW1ct was varied from 5 μM to 100 μM (Figure S9), and the UDP-Glc concentration was fixed at 1 mM. Indeed, upon increasing the concentration of HMW1ct in the ApNGT-catalyzed reaction, the final distribution of glycoforms reduced from 7-Glc to 9-Glc (5 μM HMW1ct) to 1-Glc to 5-Glc (100 μM HMW1ct). A similar trend was observed for HiNGT, although the efficiency at the lowest HMW1ct concentration (5 μM) was also greatly reduced, presumably because of the fine balance between glycosylation and inhibition of the catalytically poor HiNGT at low concentrations. Interestingly, the inhibitory effect was greatly diminished when the concentration of UDP-Glc was increased proportionally to HMW1ct (Figure S10). For both ApNGT and HiNGT, product profiles (6-Glc and 7-Glc for ApNGT, 7-Glc to 9-Glc for HiNGT) close to the fully glycosylated distribution were again observed. We hypothesize that the variation of the product distribution in response to the change in the concentration of the sugar donor may be a result of glycosylation of the most preferred sites only when UDP-Glc is limiting. In contrast, continued glycosylation of any remaining and potentially less accessible sites may occur when UDP-Glc is in excess.

Glycosylated HMW1ct Inhibits Processivity, while Early Glycoforms Efficiently Alleviate Inhibition. To obtain a better understanding of the processive fast phase of HMW1ct glycosylation, and the influence of glycosylated adhesin on processivity, a distraction assay was performed. The principle of this experiment is to test the ability of a competitor, which is typically an inhibitor or a new batch of (labeled) substrate, to distract the processive enzyme from the substrate with which it is associated. Since there are no known inhibitors of NGT glycosyltransferases, we decided to make use of the high affinity of the NGT enzymes for their glycosylated products (*vide supra*), called Glc-HMW1ct (mixture of 7,8,9,10-Glc glycoforms). Intriguingly, when the ApNGT-HMW1ct reaction (ratio 1:100) was allowed to generate early glycoforms (Figure 3A "Start" panel), the addition of Glc-HMW1ct significantly impacted the resulting product profile (Figure 3A "Distraction" panel). Whereas the control reaction quickly proceeded to produce a broad distribution of intermediate glycoforms at low levels (1-Glc to 5-Glc), the distracted reaction revealed the accumulation of 2-Glc as the major product. This change in product profile suggests that Glc-HMW1ct halts the processive phase already at the production of 2-Glc and enforces the switch to a more distributive mechanism. When the HiNGT-HMW1ct reaction (ratio 1:10) was allowed to form early glycoforms (Figure 3B, "Start" panel), the addition of Glc-HMW1ct similarly resulted in the buildup of 2-Glc and 3-Glc as the major products (Figure 3B).

Although the glycosylated product is able to prematurely halt the processive phase, still a mixture of early glycoforms is persistently produced. This suggests that the early glycoforms (1-Glc to 3-Glc) have an even higher affinity for the NGTs than both nonmodified HMW1ct and Glc-HMW1ct. The fast processive phase may be the result of the high affinity for the early glycoforms, which results in a rate enhancement in the early phases of the reaction. To test this hypothesis, an experiment was performed wherein the overnight reaction, containing mostly late glycoforms and showing only very slow

https://dx.doi.org/10.1021/acschembio.0c00848
ACS Chem. Biol. 2021, 16, 165−175

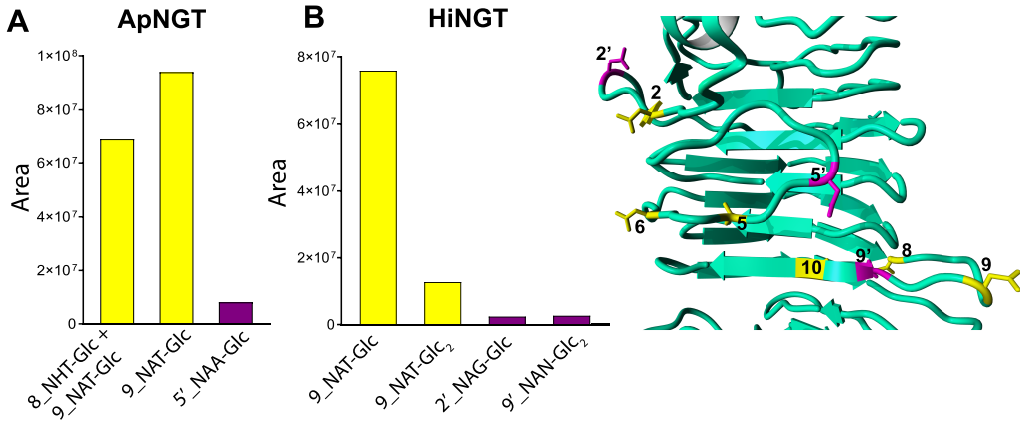


Figure 4. Preference for *N*-glycosylation sites in HMW1ct. (A) Site-specific modification for ApNGT after 0.5 min. (B) Site-specific modification for HiNGT after 0.5 min. (C) I-TASSER model of HMW1ct with sequon sites (yellow) and nonsequon sites (magenta). Representative MS spectra for specific glycosylated peptides are included in Figures S16−S19.

glycosylation, was restarted by the addition of nonglycosylated substrate (0-Glc) or early glycoforms (0-Glc to 3-Glc).

When the reaction was restarted by the addition of early glycoforms (a mixture of 0,1,2,3,4-times glycosylated HMW1ct, Figure S12C), we were intrigued to observe that the reaction proceeded at an increased rate compared to the reaction where nonmodified substrate was added (Figure S12A), producing late glycoforms in significantly shorter times as compared to the addition of nonmodified substrate only. Interestingly, in the case of HiNGT a similar trend was observed (Figure S12B,D). These results corroborate the findings above that both ApNGT and HiNGT display processive characteristics in the beginning of the reaction.

Processivity Remains under Single-Hit Conditions. As apparent from the initial time-course experiments (Figure 2), the observation of processive behavior seems influenced by the ratio of enzyme to substrate. To understand the impact of the ratio between NGT and HMW1ct, we screened several ratios of both components in a so-called "single-hit" experiment. Characteristic of a single-hit experiment is that the conditions are selected such that multiple binding events are minimized.[26,32] Generally, this is accomplished with a large substrate-to-enzyme ratio, in which case products bearing multiple modifications can only arise from persistent binding between the enzyme and product. In addition, we decided to perform these reactions under dilute conditions, to minimize inhibitory interference by the glycosylated products. Figure 3C,D show the glycoform profiles when HMW1ct was used in large excess to both ApNGT and HiNGT, resulting in enzyme/substrate ratios of 1:500 and 1:1000. Gratifyingly, in all cases the production of early glycoforms (1-Glc to 5-Glc) is apparent, which supports complex formation between NGT and HMW1ct during the first rounds of catalysis. In addition, after overnight incubation the enzymes were inhibited prematurely, generating mixtures of 2-Glc to 5-Glc in the case of ApNGT and 0-Glc to 4-Glc for HiNGT (Figures S13 and S14) highlighting the switch from the processive formation of early glycoforms to the subsequent distributive modifications, which are prevented under these single-hit conditions.

ApNGT and HiNGT Prefer Glycosylation Sites in Exposed Loops. Having established that ApNGT, and to a lesser extent HiNGT, displays processive characteristics in the initial fast phase, we wondered if NGTs in the fast phase prefer specific sites on HMW1ct. To this end, a site-preference experiment was performed in which the occupancy at all possible sites in HMW1ct was mapped by tryptic digest and LC-MS/MS at early time points. As illustrated in Figure 4A, ApNGT preferentially modifies site 9_NAT first (within the first 0.5 min of the reaction), leading to significant accumulation of the doubly glycosylated peptide (8_NHT +9_NAT), whereas sole modification of site 8_NHT was not observed. This suggests that sites 8 and 9 are modified in a processive manner, without dissociation of the enzyme between the two glycosylation events. Interestingly, also nonsequon site 5′_NAA was modified, which is situated in close proximity to sites 8 and 9, as visualized using a structural model of HMW1ct (Figure 4C, Figure S1).[33,34] After 2.5 min, especially dihexose formation at site 9_NAT appeared (Figure S15A). The site preference experiment of HiNGT (at 0.5 min) reveals a similar preference for site 9_NAT, and this site was also observed with the dihexose modification (Figure 4B). Nonsequon sites 2′_NAG and 9′_NAN were also modified, including with a dihexose in the latter case. After 20 min, modification of sites 5_NVT and 6_NTT appeared, next to dihexose formation at sites 2_NVT and 9_NAT (Figure S15B).

The model suggests that HMW1ct adopts an overall β-helix fold, which is a common architecture in bacterial autotransporter passenger domains,[20] and that all preferred sites are located on exposed loops (Figure 4C). Interestingly, although 8_NHT and 9_NAT are located in close proximity, 2_NVT and 5_NVT are situated on the other side of the HMW1ct structure. In addition, both NGTs exhibit some degree of "off-target" glycosylation, in which asparagine residues in noncanonical sequons are modified. Interestingly, these nonsequon sites are predominantly located in close proximity to the preferred sequon sites (Figure 4C), suggesting that when the enzyme is already associated, proximity will drive processive modifications. The dihexose modification may appear as a result of this proximity-induced binding, however mechanistic insight on the *O*-glycosylation step, as performed by the *N*-glycosyltransferase, is currently lacking.

ApNGT Has a Solvent-Exposed and Relaxed Acceptor Binding Site. Many structural motifs have been associated with processivity, including an extended acceptor binding site, a deep acceptor groove, a closing mechanism with part of the enzyme functioning as a lid, and a ruler helix to control product length.[22,35] Since there is no precedence for processive

https://dx.doi.org/10.1021/acschembio.0c00848
ACS Chem. Biol. 2021, 16, 165−175

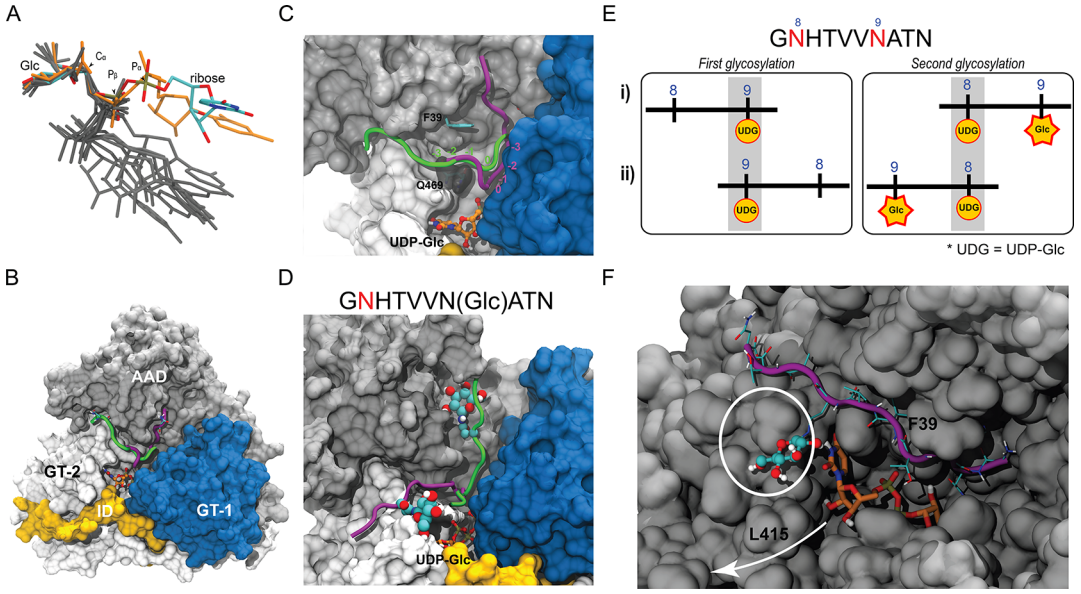


Figure 5. Docking and MD simulation of the ApNGT::peptide::UDP-Glc complex reveals relaxed acceptor binding. (A) The pyrophosphate torsion angles UDP-Glc in ApNGT (colored sticks) are more similar to the pyrophosphate angles of UDP-GlcNAc in hOGT (orange sticks), than to other glycosyltransferases in the GT-B family (gray sticks). (B) Two binding modes of peptide GN(8)HTVVN(9)ATN in ApNGT found by computational modeling presented in the purple and green cartoons (opposite N→C directions). Both peptides are bound to UDP-Glc by Asn(9) (shown in stick). (C) Close-up structure of the binding modes for the peptide GN(8)HTVVN(9)ATN, docked to UDP-Glc via Asn(9). (D) Close-up structure of the binding modes for the peptide GN(8)HTVVN(Glc)ATN, docked to UDP-Glc via Asn(8). (E) Schematic representation of the possible mechanisms in which the peptide is docked at site Asn(9), is glycosylated, and then slides in the forward direction (i) to allow glycosylation at site Asn(8), or in the reversed direction (ii). (F) Space-filling model of the ApNGT::Glc-peptide::UDP-Glc complex that suggests there is enough space for UDP to dissociate and UDP-Glc to associate in between glycosylation events.

character in monomeric protein glycosyltransferases, we set out to identify the possible structural elements that are responsible for processivity using docking and molecular dynamics (MD) simulations. We selected ApNGT because there is one report of a crystal structure with UDP bound (PDB: 3Q3H).[36] First the glucose was added to generate a docked structure of ApNGT::UDP-Glc, which was used as a scaffold for peptide docking. The similarities between hOGT and ApNGT are evident when comparing UDP-GlcNAc and UDP-Glc, respectively (Figure 5A), to nucleotide-sugar conformations from several other complexes within the GT-B enzyme family (i.e., inverting enzymes MurG, UGT71G1, UGT72B1, VvGT1, and retaining enzymes AGT, OtsA, WaaG).[37] The unusual UDP-sugar pyrophosphate conformation positions the α-phosphate to act as the proton acceptor in the hOGT-catalyzed glycosylation reaction.[37] In this regard, the pyrophosphate torsion angles of UDP-Glc are more similar to the angles of UDP-GlcNAc in hOGT than to the angles of all the other nucleotide-sugar structures. Protein−ligand interactions in the UDP-sugar binding site resemble those observed in hOGT (Figure S20).

Next, the complex of ApNGT::UDP-Glc with the peptide GN(8)HTVVN(9)ATN (corresponding to HMW1ct sequons 8 and 9) was created to assess possible binding poses of the preferred adhesin fragment (Figure 5B). The nucleophilic N from Asn(9) was constrained to be in close proximity to the anomeric C_α carbon, and peptide binding modes were generated. The binding site of ApNGT was found to be flexible enough to allow several peptide binding modes (Figure 5C, main binding modes in green and purple) near the postulated acceptor binding groove and making contacts with the proposed acceptor binding residues Phe39, His272, His277, and Gln469.[36,38] Our results suggest that the peptide-binding region in ApNGT is located on the solvent-exposed enzyme surface. In contrast, in hOGT the unfolded peptide binds in a groove that is located inside a superspiral formed by repeated TPR regions.[39] The known crystal structures of hOGT show two binding modes either with a shallow pose (Figure S21, purple cartoon) or more embedded pose in the TPR domain (Figure S21, green cartoon), where the former recognizes semifolded peptide regions, and the latter is for extended peptides.[39] Interestingly, ApNGT revealed unexpected flexibility in peptide binding, and opposing orientations with respect to the *N*- and *C*-termini appeared to bind stably (Figure 5C). In contrast, the crystal structures of hOGT show the peptides in only one orientation (Figure S21).

As the experimental data suggest that one Glc modification promotes a second Glc-transfer, we generated peptide−enzyme complexes with the glycosylated peptide GN(8)-HTVVN(Glc)ATN, with preferred site 9_NAT glycosylated (*vide supra*). Two regions for the binding of the Glc moiety were found (Figure 5D, space-filling models), but none of these displayed increased affinities. Interestingly, the Interface Score of the peptide−protein complex, with and without glycosylation, was around −35 kcal/mol, suggesting similar binding energies for both peptide and Glc-peptide. MD simulations of the Glc-peptide complex did not show Glc-focused interactions with ApNGT. On the basis of the computational modeling, we hypothesize that after glycosylation of the first site (N(9)AT), the peptide slides along the enzyme to achieve a second glycosylation at N(8)HT, while anchoring to the enzyme with its N(Glc)AT site (Figure 5E). Because ApNGT is flexible in the N- to C-terminus direction that the peptide binds, this process could potentially happen in the opposite direction. In addition, the model suggests there is

https://dx.doi.org/10.1021/acschembio.0c00848
ACS Chem. Biol. 2021, 16, 165−175

enough space for UDP available to dissociate and be substituted for a new UDP-Glc, to continue catalysis (Figure 5F).

DISCUSSION

Protein glycosyltransferases are abundantly present in all domains of life, and are found to catalyze a wide range of protein modifications, with new examples emerging at a steady pace.[40] They show an intriguing level of diversity in specificity for both sugar donors and protein substrates but also recognition elements (amino acid residues, structural folds) and timing of modification (co- or post-translational). As protein glycosylation is not genetically encoded, the spatiotemporal drivers and effects of protein glycosylation are at the same time exciting and challenging to study.

Our results reveal how ApNGT, and to a lesser extent HiNGT, perform hyperglycosylation of HMW1ct adhesin in a two-phase mechanism (Figure 6). In the beginning of the

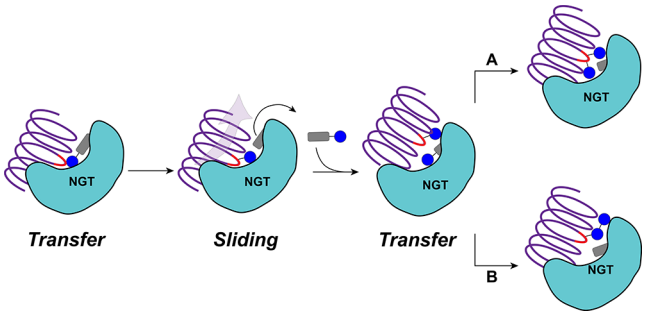


Figure 6. Model for the sliding mechanism in the fast phase in the semiprocessive glycosylation of HMW1ct by NGTs that results in processive glycosylation of adjacent sites (A) and dihexose formation (B). Blue circle = glucose; gray rectangle = UDP.

reaction, ApNGT glycosylates HMW1ct using a processive mechanism that yields a broad distribution of intermediate glycoforms. Compared to the starting substrate HMW1ct, especially the early glycoforms seem to be suitable substrates for processive modification, which is a characteristic of processive enzymes. However, the enzyme−substrate complex is receptive to the presence of the fully modified Glc-HMW1ct product that successfully competes with binding to the enzyme, resulting in a shortening of the processive phase. After this fast processive phase, both ApNGT and HiNGT are increasingly inhibited by the high affinity for the glycosylated product Glc-HMW1ct and only incrementally add glucose residues to remaining sites. The fact that dihexose formation and modification of nonsequon sites generally happens on and in close proximity to defined sequons further strengthens the hypothesis that NGTs employ proximity-induced processive glycosylation. However, whether NGTs stay fully associated to ensure processivity or they engage in "hopping" (i.e., microscopic dissociation followed by quick reassociation), in analogy to processivity in DNA-binding proteins, is currently impossible to determine.[41,42] A hallmark of processivity is the high affinity of the enzyme for its product. Therefore, processive enzymes may be more sensitive to product inhibition than enzymes that employ a distributive mechanism.[43] Conversely, because distributive enzymes dissociate after catalysis, they may also be susceptible to competitor binding. For distributive protein kinases, an increase in substrate concentration results in accumulation of partially phosphorylated species, that serve as competitive kinase inhibitors.[30] As the NGTs studied here display characteristics of both processes, we suggest denoting the mechanism of these NGTs as semiprocessive. We propose a mechanistic model that starts with NGT binding to HMW1ct, followed by fast and processive glycosylation of adjacent sites facilitated by sliding over the NGT surface (Figure 6A) or dihexose formation (Figure 6B). We expect that this promiscuous surface binding is a structural basis for processivity, as the lack thereof may be at the basis of the distributive character observed in hOGT.[23,36] After a few additional modifications, NGT enters a slower distributive phase, in which it may randomly bind to both sequon and nonsequon sites on the surface of HMW1ct. The resulting products have high affinity for the NGTs, resulting in retardation of glycosylation by product inhibition. Together, this leads us to propose a semiprocessive mechanism for NGTs.

There are other reports of glycosyltransferases that operate through a two-phase mechanism, both in the process of carbohydrate polymerization and in protein hyperglycosylation. For homogalacturonan polysaccharide synthesis, a clear distinction was observed between enzyme activity on short (DP $\leq$ 7) and long acceptor substrates (DP $\geq$ 11), resulting in two kinetic phases that display both distributive and processive character.[57] In addition, *O*-glycosylation of GspB adhesin proteins in *Streptococcus gordonii* is catalyzed by a tetrameric GtfA/GtfB complex that has distinct kinetic profiles on nonmodified and partially modified substrates.[58] Whereas the initial modifications are occurring rapidly (fast phase), the ensuing glycosylation events appear at a lower rate (slow phase), presumably as a result of a change in enzyme complex architecture in response to increased glycosylation.

The observed product inhibition of NGT and concomitant switch to a distributive mechanism of glycosylation may be induced by our *in vitro* setup. In the natural systems, the glycosylated proteins are typically exported outside of the cell using a transport system, of which the timing and cellular location may have an impact on the concentrations of NGT and acceptor substrate. This is in analogy to the mechanistic differences reported for bacterial membrane-associated polysialyltransferases that revealed a nonprocessive mechanism *in vitro*, and a processive mechanism *in vivo*.[59,60]

Interestingly, the C-terminal part of the HMW1A adhesin (~ 330 amino acids) that we used as a model protein in our study is reported to display only three Glc residues *in vivo*.[18] The majority of Glc residues in the native HMW1A adhesin (~1530 amino acids) appear on the N-terminal part, where 46 hexose residues are found on 31 sites.[18] This discrepancy in Glc loading in the C-terminal fragment may be explained by poor accessibility of this part in the native system as it is supposedly close to the cell membrane. It will be highly insightful to investigate the mechanism of hyperglycosylation on the full HMW1A adhesin protein.

NGTs have a high preference for sequons that are exposed on the surface of the acceptor protein, which is consistent with the post-translational timing of the modification. Moreover, especially the bacterial adhesins and autotransporters share a general β-helical fold,[44,45] which is also highly associated with two-partner secretion proteins in different species.[46,47] It will be highly revealing to investigate other known and predicted NGTs for processive characteristics[48] and revisit currently known β-helical adhesins to find an associated NGT.

https://dx.doi.org/10.1021/acschembio.0c00848
ACS Chem. Biol. 2021, 16, 165−175

The clear processive features in the NGTs under study here raises the question of the functional relevance. Processivity is well-established in template-driven production of oligonucleotides. For post-translational modifications, such as phosphorylation and glycosylation, there is little knowledge on the importance of multisite modifications, but the sheer number of modifications may seem more important than the specific locations. The high association rate of the substrate and processivity of early glycoforms may ensure a high level of Glc-modifications on the HMW adhesins before export by the HMW1B translocator. In general, the density of epitopes is directly linked to the efficiency of natural multivalent interactions and is proposed to serve as a mechanism to regulate the biological interaction.[49] Multisite glycosylation may be an elegant solution to ensure efficient bacterial attachment to receptors through multivalency,[50−52] to overcome the generally poor (mM range) affinity of proteins for carbohydrate ligands.

The knowledge that NGTs can support processive characteristics is important in the biotechnological use of such enzymes to create well-defined glycoproteins. Several studies have focused on employing NGTs (and their engineered variants) in the biosynthesis of defined glycoproteins for biotechnological applications and vaccine development.[38,48,53−55] The ApNGT mutant Q469A showed reduced product inhibition and produced a more homogeneously glycosylated HMW1ct, with up to 10 residues. On the basis of the central position of Q469 in both UDP-Glc and peptide binding as revealed by molecular modeling, we propose that Q469 may function as a "processive switch," preventing the glycosylated product from leaving the binding site, and thereby increasing the association required for an additional round of catalysis.[38] Sequence alignment indicates a corresponding Gln residue in a conserved region in HiNGT (Gln495, Figure S22), but without more structural information, it is difficult to assess its involvement in the mechanism.

Our results suggest that glycoprotein production systems based on NGT expression in *E. coli* may suffer from low UDP-Glc levels (typically, 1−2 mM),[56] as that may lead to premature product inhibition. In agreement with other reports,[31] we found that the glycosylation of HMW1ct is highly dependent on the levels of NGTs. As we show that processivity in NGTs arises from their high affinity for the intermediate products, we expect that this may inspire a class of inhibitors that capitalize on product binding, for instance by generating glycosylated β-helical peptide scaffolds.

In summary, we have provided evidence that both ApNGT and HiNGT display processive characteristics in the first fast phase of HMW1ct glycosylation, followed by a phase with distributive features, together resulting in a semiprocessive mechanism. Molecular modeling reveals that ApNGT has promiscuous substrate binding preference, which allows for sliding of the enzyme along the adhesin surface. Further investigations into the mechanisms of other bacterial NGTs will reveal whether processivity is a general mechanism that bacteria use to achieve hyperglycosylation of extracellular proteins involved in virulence.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00848.

Description of the experimental procedures, supplementary figures and tables, whole protein MS and MS/MS spectra, processivity parameter calculations, kinetic analysis curves, SPR sensograms, and docking results; molecular simulation files made available by the corresponding author upon request (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Marthe T. C. Walvoort − *Stratingh Institute for Chemistry, University of Groningen, 9747, AG, Groningen, The Netherlands;* orcid.org/0000-0003-1101-2659; Email: m.t.c.walvoort@rug.nl

Authors

Liubov Yakovlieva − *Stratingh Institute for Chemistry, University of Groningen, 9747, AG, Groningen, The Netherlands;* orcid.org/0000-0003-2061-4352

Carlos Ramírez-Palacios − *Groningen Biomolecular Sciences and Biotechnology Institute, 9747, AG, Groningen, The Netherlands*

Siewert J. Marrink − *Groningen Biomolecular Sciences and Biotechnology Institute, 9747, AG, Groningen, The Netherlands;* orcid.org/0000-0001-8423-5277

Complete contact information is available at: https://pubs.acs.org/10.1021/acschembio.0c00848

Author Contributions

L.Y. and M.T.C.W. conceived the project. L.Y., S.J.M., and M.T.C.W. designed and coordinated the study. The biochemical experiments were performed by L.Y., and the docking and simulations were performed by C.R.P. L.Y., C.R.P., and M.T.C.W. wrote the manuscript, with input from all authors. All authors were involved in proofreading the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Interfaculty Mass Spectrometry Center of the University of Groningen (H. P. Permentier and M. P. de Vries) for help with whole-protein mass analysis, J. Hekelaar and M. Rovetta for their assistance with the site-preference experiments, R. H. Cool for help with the SPR analyses. We thank the Center for Information Technology of the University of Groningen for providing access to the Peregrine high-performance computing cluster. We thank B. Imperiali and G. Maglia for insightful discussions and proofreading of the manuscript. This work was financially supported by the Dutch Organization for Scientific Research (VENI 722.016.006) and the European Union through the Rosalind Franklin Fellowship COFUND project 60021 (both to M.T.C.W.) and by a CONACYT doctoral fellowship (to C.R.P.).

■ REFERENCES

(1) Varki, A. (2017) Biological roles of glycans. *Glycobiology 27*, 3.
(2) Pinho, S. S., and Reis, C. A. (2015) Glycosylation in cancer: mechanisms and clinical implications. *Nat. Rev. Cancer 15*, 540.
(3) Bennett, E. P., Mandel, U., Clausen, H., Gerken, T. A., Fritz, T. A., and Tabak, L. A. (2012) Control of mucin-type O-glycosylation: A classification of the polypeptide GalNAc-transferase gene family. *Glycobiology 22*, 736.
(4) Yao, Q., Lu, Q. H., Wan, X. B., Song, F., Xu, Y., Hu, M., Zamyatina, A., Liu, X. Y., Huang, N., Zhu, P., and Shao, F. (2014) A

structural mechanism for bacterial autotransporter glycosylation by a dodecameric heptosyltransferase family. *eLife 3*, No. e03714.
(5) Bai, L., Wang, T., Zhao, G. P., Kovach, A., and Li, H. L. (2018) The atomic structure of a eukaryotic oligosaccharyltransferase complex. *Nature 555*, 328.
(6) Napiorkowska, M., Boilevin, J., Darbre, T., Reymond, J. L., and Locher, K. P. (2018) Structure of bacterial oligosaccharyltransferase PglB bound to a reactive LLO and an inhibitory peptide. *Sci. Rep. 8*, 16297.
(7) Kreppel, L. K., Blomberg, M. A., and Hart, G. W. (1997) Dynamic glycosylation of nuclear and cytosolic proteins - Cloning and characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats. *J. Biol. Chem. 272*, 9308.
(8) Caval, T., Tian, W. H., Yang, Z., Clausen, H., and Heck, A. J. R. (2018) Direct quality control of glycoengineered erythropoietin variants. *Nat. Commun. 9*, 3342.
(9) Hart, G. W., Slawson, C., Ramirez-Correa, G., and Lagerlof, O. (2011) Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. *Annu. Rev. Biochem. 80*, 825.
(10) Grass, S., Lichti, C. F., Townsend, R. R., Gross, J., and St. Geme, J. W. (2010) The *Haemophilus influenzae* HMW1C protein is a glycosyltransferase that transfers hexose residues to asparagine sites in the HMW1 adhesin. *PLoS Pathog. 6* (5), No. e1000919.
(11) Grass, S., Buscher, A. Z., Swords, W. E., Apicella, M. A., Barenkamp, S. J., Ozchlewski, N., and St Geme, J. W. (2003) The *Haemophilus influenzae* HMW1 adhesin is glycosylated in a process that requires HMW1C and phosphoglucomutase, an enzyme involved in lipooligosaccharide biosynthesis. *Mol. Microbiol. 48*, 737.
(12) Choi, K.-J., Grass, S., Paek, S., St. Geme, J. W., III, and Yeo, H.-J. (2010) The *Actinobacillus pleuropneumoniae* HMW1C-like glycosyltransferase mediates N-linked glycosylation of the *Haemophilus influenzae* HMW1 adhesin. *PLoS One 5*, e15888.
(13) Schwarz, F., Fan, Y.-Y., Schubert, M., and Aebi, M. (2011) Cytoplasmic N-glycosyltransferase of *Actinobacillus pleuropneumoniae* is an inverting enzyme and recognizes the NX(S/T) consensus sequence. *J. Biol. Chem. 286*, 35267.
(14) Rempe, K. A., Spruce, L. A., Porsch, E. A., Seeholzer, S. H., Norskov-Lauritsen, N., and St. Geme, J. W., III. (2015) Unconventional N-linked glycosylation promotes trimeric autotransporter function in *Kingella kingae* and *Aggregatibacter aphrophilus*. *mBio 6*, 1206.
(15) Campbell, J. A., Davies, G. J., Bulone, V., and Henrissat, B. A. (1997) Classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochem. J. 326*, 929.
(16) Coutinho, P. M., Deleury, E., Davies, G. J., and Henrissat, B. (2003) An evolving hierarchical family classification for glycosyltransferases. *J. Mol. Biol. 328*, 307.
(17) Naegeli, A., Neupert, C., Fan, Y. Y., Lin, C. W., Poljak, K., Papini, A. M., Schwarz, F., and Aebi, M. (2014) Molecular analysis of an alternative N-glycosylation machinery by functional transfer from *Actinobacillus pleuropneumoniae* to *Escherichia coli*. *J. Biol. Chem. 289*, 2170.
(18) Gross, J., Grass, S., Davis, A. E., Gilmore-Erdmann, P., Townsend, R. R., and St. Geme, J. W. (2008) The *Haemophilus influenzae* HMW1 adhesin is a glycoprotein with an unusual N-linked carbohydrate modification. *J. Biol. Chem. 283*, 26010.
(19) Osman, K. L., Jefferies, J. M., Woelk, C. H., Cleary, D. W., and Clarke, S. C. (2018) The adhesins of non-typeable *Haemophilus influenzae*. *Expert Rev. Anti-Infect. Ther. 16*, 187.
(20) Nishimura, K., Tajima, N., Yoon, Y. H., Park, S. Y., and Tame, J. R. H. (2010) Autotransporter passenger proteins: virulence factors with common structural themes. *J. Mol. Med. 88*, 451.
(21) Walvoort, M. T. C., Testa, C., Eilam, R., Aharoni, R., Nuti, F., Rossi, G., Real-Fernandez, F., Lanzillo, R., Brescia Morra, V., Lolli, F., Rovero, P., Imperiali, B., and Papini, A. M. (2016) Antibodies from multiple sclerosis patients preferentially recognize hyperglucosylated adhesin of non-typeable *Haemophilus influenzae*. *Sci. Rep. 6*, 39430.
(22) Yakovlieva, L., and Walvoort, M. T. C. (2020) Processivity in bacterial glycosyltransferases. *ACS Chem. Biol. 15*, 3.
(23) Lu, L., Fan, D. C., Hu, C. W., Worth, M., Ma, Z. X., and Jiang, J. Y. (2016) Distributive O-GlcNAcylation on the highly repetitive C-terminal domain of RNA polymerase II. *Biochemistry 55*, 1149.
(24) Horn, S. J., Sørlie, M., Varum, K. M., Valjamae, P., and Eijsink, V. G.H. (2012) Measuring processivity. *Methods Enzymol. 510*, 69.
(25) Washington, M. T., Johnson, R. E., Prakash, S., and Prakash, L. (1999) Fidelity and processivity of *Saccharomyces cerevisiae* DNA polymerase *η*. *J. Biol. Chem. 274*, 36835.
(26) Levengood, M. R., Splain, R. A., and Kiessling, L. L. (2011) Monitoring processivity and length control of a carbohydrate polymerase. *J. Am. Chem. Soc. 133*, 12758.
(27) McClure, W. R., and Chow, Y. (1980) In *Methods in Enzymology* (Purich, D. L., Ed.) Vol. *64*, Academic Press.
(28) Pellicena, P., and Miller, W. T. (2001) Processive phosphorylation of p130Cas by Src depends on SH3-polyproline interactions. *J. Biol. Chem. 276*, 28190.
(29) Patwardhan, P., and Miller, W. T. (2007) Processive phosphorylation: Mechanism and biological importance. *Cell. Signalling 19*, 2218.
(30) Burack, W. R., and Sturgill, T. W. (1997) The activating dual phosphorylation of MAPK by MEK is nonprocessive. *Biochemistry 36*, 5929.
(31) Elango, D., and Schulz, B. L. (2020) Phase-variable glycosylation in nontypeable *Haemophilus influenzae*. *J. Proteome Res. 19*, 464.
(32) Bambara, R. A., Fay, P. J., and Mallaber, L. M. (1995) Methods of analyzing processivity. *Methods Enzymol. 262*, 270.
(33) Yang, J. Y., and Zhang, Y. (2015) I-TASSER server: new development for protein structure and function predictions. *Nucleic Acids Res. 43*, W174.
(34) Yang, J. Y., Yan, R. X., Roy, A., Xu, D., Poisson, J., and Zhang, Y. (2015) The I-TASSER Suite: protein structure and function prediction. *Nat. Methods 12*, 7.
(35) Breyer, W. A., and Matthews, B. W. (2001) A structural basis for processivity. *Protein Sci. 10*, 1699.
(36) Kawai, F., Grass, S., Kim, Y., Choi, K. J., St. Geme, J. W., and Yeo, H. J. (2011) Structural insights into the glycosyltransferase activity of the *Actinobacillus pleuropneumoniae* HMW1C-like protein. *J. Biol. Chem. 286*, 38546.
(37) Schimpl, M., Zheng, X. W., Borodkin, V. S., Blair, D. E., Ferenbach, A. T., Schuttelkopf, A. W., Navratilova, I., Aristotelous, T., Albarbarawi, O., Robinson, D. A., Macnaughtan, M. A., and van Aalten, D. M. F. (2012) O-GlcNAc transferase invokes nucleotide sugar pyrophosphate participation in catalysis. *Nat. Chem. Biol. 8*, 969.
(38) Song, Q. T., Wu, Z. G., Fan, Y. Y., Song, W. R., Zhang, P. R., Wang, L., Wang, F. X., Xu, Y. Y., Wang, P. G., and Cheng, J. S. (2017) Production of homogeneous glycoprotein with multisite modifications by an engineered N-glycosyltransferase mutant. *J. Biol. Chem. 292*, 8856.
(39) Pathak, S., Alonso, J., Schimpl, M., Rafie, K., Blair, D. E., Borodkin, V. S., Schuttelkopf, A. W., Albarbarawi, O., and van Aalten, D. M. F. (2015) The active site of O-GlcNAc transferase imposes constraints on substrate sequence. *Nat. Struct. Mol. Biol. 22*, 744.
(40) Lassak, J., Keilhauer, E. C., Furst, M., Wuichet, K., Godeke, J., Starosta, A. L., Chen, J. M., Sogaard-Andersen, L., Rohr, J., Wilson, D. N., Häussler, S., Mann, M., and Jung, K. (2015) Arginine-rhamnosylation as new strategy to activate translation elongation factor P. *Nat. Chem. Biol. 11*, 266.
(41) Komazin-Meredith, G., Mirchev, R., Golan, D. E., van Oijen, A. M., and Coen, D. M. (2008) Hopping of a processivity factor on DNA revealed by single-molecule assays of diffusion. *Proc. Natl. Acad. Sci. U. S. A. 105*, 10721.
(42) Halford, S. E., and Marko, J. F. (2004) How do site-specific DNA-binding proteins find their targets? *Nucleic Acids Res. 32*, 3040.
(43) Bu, L. T., Nimlos, M. R., Shirts, M. R., Stahlberg, J., Himmel, M. E., Crowley, M. F., and Beckham, G. T. (2012) Product binding

varies dramatically between processive and nonprocessive cellulase enzymes. *J. Biol. Chem. 287*, 24807.
(44) Lu, Q. H., Yao, Q., Xu, Y., Li, L., Li, S., Liu, Y. H., Gao, W. Q., Niu, M., Sharon, M., Ben-Nissan, G., Zamyatina, A., Liu, X., Chen, S., and Shao, F. (2014) An iron-containing dodecameric heptosyltransferase family modifies bacterial autotransporters in pathogenesis. *Cell Host Microbe 16*, 351.
(45) Yeo, H. J., Yokoyama, T., Walkiewicz, K., Kim, Y., Grass, S., and Geme, J. W. (2007) The structure of the *Haemophilus influenzae* HMW1 pro-piece reveals a structural domain essential for bacterial two-partner secretion. *J. Biol. Chem. 282*, 31076.
(46) ur Rahman, S., and van Ulsen, P. (2013) System specificity of the TpsB transporters of coexpressed two-partner secretion systems of *Neisseria meningitidis*. *J. Bacteriol. 195*, 788.
(47) Baelen, S., Dewitte, F., Clantin, B., and Villeret, V. (2013) Structure of the secretion domain of HxuA from *Haemophilus influenzae*. *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun. 69*, 1322.
(48) Lin, L., Kightlinger, W., Prabhu, S. K., Hockenberry, A. J., Li, C., Wang, L. X., Jewett, M. C., and Mrksich, M. (2020) Sequential glycosylation of proteins with substrate-specific N-glycosyltransferases. *ACS Cent. Sci. 6*, 144.
(49) Cairo, C. W., Gestwicki, J. E., Kanai, M., and Kiessling, L. L. (2002) Control of multivalent interactions by binding epitope density. *J. Am. Chem. Soc. 124*, 1615.
(50) Kiessling, L. L., Gestwicki, J. E., and Strong, L. E. (2000) Synthetic multivalent ligands in the exploration of cell-surface interactions. *Curr. Opin. Chem. Biol. 4*, 696.
(51) Bernardi, A., Jimenez-Barbero, J., Casnati, A., De Castro, C., Darbre, T., Fieschi, F., Finne, J., Funken, H., Jaeger, K. E., Lahmann, M., et al. (2013) Multivalent glycoconjugates as anti-pathogenic agents. *Chem. Soc. Rev. 42*, 4709.
(52) Lundquist, J. J., and Toone, E. J. (2002) The cluster glycoside effect. *Chem. Rev. 102*, 555.
(53) Kightlinger, W., Lin, L., Rosztoczy, M., Li, W. H., DeLisa, M. P., Mrksich, M., and Jewett, M. C. (2018) Design of glycosylation sites by rapid synthesis and analysis of glycosyltransferases. *Nat. Chem. Biol. 14*, 627.
(54) Tytgat, H. L. P., Lin, C.-w., Levasseur, M. D., Tomek, M. B., Rutschmann, C., Mock, J., Liebscher, N., Terasaka, N., Azuma, Y., Wetter, M., Bachmann, M. F., Hilvert, D., Aebi, M., and Keys, T. G. (2019) Cytoplasmic glycoengineering enables biosynthesis of nanoscale glycoprotein assemblies. *Nat. Commun. 10*, 5403.
(55) Fulton, K. M., Smith, J. C., and Twine, S. M. (2016) Clinical applications of bacterial glycoproteins. *Expert Rev. Proteomics 13*, 345.
(56) Mao, Z. C., Shin, H. D., and Chen, R. R. Z. (2006) Engineering the E. coli UDP-glucose synthesis pathway for oligosaccharide synthesis. *Biotechnol. Prog. 22*, 369.
(57) Amos, R. A., Pattathil, S., Yang, J. − Y., Atmodjo, M. A., Urbanowicz, B. R., Moremen, K. W., and Mohnen, D. (2018) A two-phase model for the non-processive biosynthesis of homogalacturonan polysaccharides by the GAUT1:GAUT7 complex. *J. Biol. Chem. 293*, 19047−19063.
(58) Chen, Y., Seepersaud, R., Bensing, B. A., Sullam, P. M., and Rapoport, T. A. (2016) Mechanism of a cytosolic *O*-glycosyltransferase essential for the synthesis of a bacterial adhesion protein. *Proc. Natl. Acad. Sci. U. S. A. 113*, E1190−E1199.
(59) Freiberger, F., Claus, H., Günzel, A., Oltmann-Norden, I., Vionnet, J., Mühlenhoff, M., Vogel, U., Vann, W. F., Gerardy-Schahn, R., and Stummeyer, K. (2007) Biochemical characterization of a *Neisseria meningitidis* polysilyltransferase reveals novel functional motifs in bacterial sialyltransferases. *Mol. Microbiol. 65*, 1258−1275.
(60) Steenbergen, S. M., and Vimr, E. R. (2003) Functional relationships of the sialyltransferases involved in expression of the polysialic acid capsules of *Escherichia coli* K1 and K92 and *Neisseria meningitidis* Groups B or C. *J. Biol. Chem. 278*, 15349−15359.

https://dx.doi.org/10.1021/acschembio.0c00848
ACS Chem. Biol. 2021, 16, 165−175