The action of *N*-acetylglucosaminyltransferase-V is prevented by the bisecting GlcNAc residue at the catalytic step

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Abstract Using a purified protein and bisected acceptor oligosaccharides, we demonstrate that *N*-acetylglucosaminyltransferase (GnT)-V transfers a *N*-acetylglucosamine residue via a β1,6-linkage to the bisected oligosaccharides. We also kinetically characterized the substrate specificity of GnT-V with respect to the bisected oligosaccharide. Although the K_m values for the bisected acceptors were comparable to that for a non-bisected acceptor, the V_{max} values for the bisected acceptors were much lower than that for the non-bisected acceptor. These findings suggest that the acceptor specificity of GnT-V is determined by the catalytic process rather than by its binding to the substrate. It was also found that the presence of the 2-*N*-acetyl group in the bisecting monosaccharide moiety plays a critical role in determining the catalytic efficiency of the enzyme.

Key words: Bisecting *N*-acetylglucosamine; β1,6-Branched *N*-acetylglucosamine; Kinetic analysis; *N*-Acetylglucosaminyltransferase-V; *N*-Glycan

1. Introduction

The bisecting *N*-acetylglucosamine (GlcNAc) moiety, catalyzed by *N*-acetylglucosaminyltransferase (GnT)-III, is known to be a unique structure, because asparagine-linked glycans (*N*-glycans) with a bisecting GlcNAc moiety do not serve as acceptor substrates for α-mannosidase II and GnTs-II, -IV, and -V, and, thus, the formation of additional branching is inhibited [1–6]. As a result, the action of GnT-III prior to GnT-IV and -V leads to the inhibition of the biosynthesis of multi-antennary oligosaccharides. In addition to its importance in the biosynthesis of sugar chains, the significance of the bisecting GlcNAc has been implicated in a variety of other biological events. The biological significance of the effect of the bisecting GlcNAc on inhibiting β1,6-branch formation has been extensively investigated. It has been proposed that the β1,6-branching structure produced by GnT-V might be associated with malignant transformation and tumor metastasis [7–11]. The overexpression of GnT-III in highly metastatic melanoma cells led to a reduction in the number of β1,6-branches in cell surface *N*-glycans, with a parallel increase in bisected oligosaccharides, and a suppression of lung metastasis of the melanoma cells [12]. Therefore, the mechanism of how bisected oligosaccharides prevent additional branching formation is an important issue. Nuclear magnetic resonance (NMR) studies suggest that the addition of a bisecting GlcNAc caused a drastic alteration in the overall conformation of the *N*-glycans [13,14]. Furthermore, the presence of a bisecting GlcNAc has a significant effect on conformation of the α1,6-linked mannose (Man) moiety [15], and, therefore, it has been believed that the prevention of β1,6-branching by the addition of a bisecting GlcNAc is due, at least in part, to the induction of a conformational change in the *N*-glycans. However, analyses of the kinetics of GnT-V toward the bisected acceptor have not been investigated, probably due to the limited availability of the purified enzyme. A large amount of pure GnT-V is required to produce a detectable amount of the reaction product, when bisected oligosaccharides are used as acceptors.

In the present study, the acceptor specificity of GnT-V was kinetically characterized by using a purified recombinant enzyme [16] and bisected acceptor substrates [17]. Our findings show that GnT-V binds bisected acceptors at the same level as non-bisected acceptors but transfers a GlcNAc residue via a β1,6-linkage at a very low level. Thus, the specificity of GnT-V with respect to the acceptor oligosaccharides appears to be critically determined not during binding with the acceptor oligosaccharides but during the catalytic process.

2. Materials and methods

2.1. Materials

UDP-GlcNAc and UDP-glucose (Glc) were obtained from Sigma. The standard oligosaccharides were purchased from Takara. Glycosidases were obtained from the following sources: sialidase (*Arthrobacter ureafacies*) from Nacalai Tesque; β-galactosidase (jack bean) from Seikagaku; α-fucosidase (bovine kidney) from Sigma. Other common chemicals were obtained from Wako Pure Chemicals.

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Abbreviations: GlcNAc and Gn, *N*-acetylglucosamine; GnT, *N*-acetylglucosaminyltransferase; *N*-glycan, asparagine-linked glycan; Man, mannose; Glc, glucose; PA, 2-aminopyridine; GnGn-bi-PA, PA-labeled agalactobiantennary oligosaccharide; HPLC, high performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; Gn-bisected bi-PA, PA-labeled agalactobiantennary sugar chain with bisecting GlcNAc; Glc-bisected bi-PA, PA-labeled agalactobiantennary sugar chain with bisecting Glc

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2.2. Preparation of purified enzyme

The C-terminal histidine-tagged recombinant GnT-V, which contains the catalytic region (Tyr188–Leu741) of human GnT-V, was completely purified as described previously [16]. Protein concentrations were determined using the method described by Bradford using bovine serum albumin as a standard [18].

2.3. Preparation of acceptor substrates

A disialyl biantennary oligosaccharide was isolated from hen's egg yolk, as described previously [19]. The resulting free glycan was sequentially digested with sialidase, β-galactosidase, and α-fucosidase, in this order, and the resulting glycan was then labeled with 2-aminopyridine (PA), as reported previously [20]. This obtained PA-labeled agalactobiantennary oligosaccharide (GnGn-bi-PA) was used as a non-bisected acceptor. Two types of bisected acceptors were obtained from the non-bisected acceptor via catalysis by GnT-III using UDP-GlcNAc and UDP-Glc as donor substrates [17]. The reaction products were then collected and concentrated. The concentrations of these acceptor oligosaccharides were determined by the amount of the PA group using a known amount of GnGn-bi-PA as a standard.

2.4. GnT-V activity assay

The GnT-V activity was assayed as described previously [21]. Purified enzyme was incubated in 125 mM MES–NaOH (pH 6.25) containing 200 mM GlcNAc, 0.5% Triton X-100 and 10 mM EDTA at 37°C, in the presence of acceptors and UDP-GlcNAc. The reaction was terminated by heating the mixture at 100°C for 2 min, and the sample was then centrifuged at 15 000 rpm for 5 min in a microfuge. The resulting supernatant was analyzed by reversed phase high performance liquid chromatography (HPLC) (Shimadzu) using a TSKgel ODS-80TM (4.6×150, Tosoh). The solvent used was a 20 mM ammonium acetate buffer (pH 4.0) containing 0.1% 1-butanol. The substrate and the product were isocratically separated, and fluorescence was detected with a fluorescence detector (RF-10AXL, Shimadzu) at excitation and emission wavelengths of 320 nm and 400 nm, respectively.

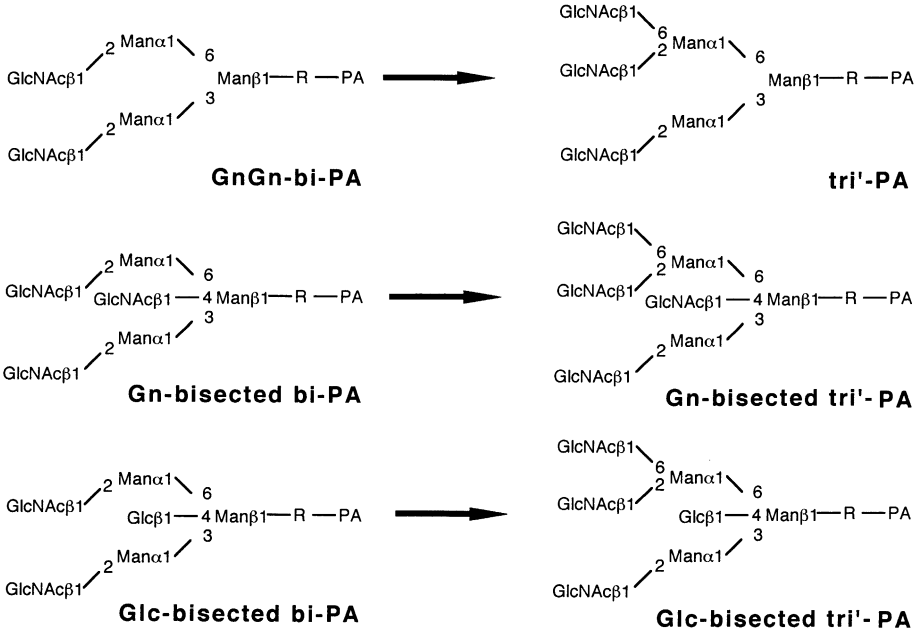
2.5. Electrospray ionization mass spectrometry (ESI-MS)

ESI-MS was carried out as described previously [16,17] using an LCQ (Finnigan) quadrupole mass spectrometer. The PA-labeled oligosaccharide was dissolved in 50% aqueous methanol and introduced into the ion source by direct infusion at a flow rate of 3 μl/min using a syringe pump interfaced with the system. ESI-MS spectra were obtained using the positive ion mode. The ion spray voltage and capillary voltage were 4.5 kV and 10 V, respectively, and the capillary temperature was 200°C. Full scan spectra were obtained in the range of 1200–2000 of *m/z* values.

3. Results and discussion

A purified recombinant GnT-V was produced using a baculovirus–insect cell system [16] as an enzyme source and GnGn-bi-PA (Scheme 1) as the acceptor substrate [21]. In order to examine the issue of whether GnT-V transfers a GlcNAc residue to the bisected acceptors, two types of PA-labeled oligosaccharides were employed: Gn-bisected bi-PA and Glc-bisected bi-PA (Scheme 1). These bisected acceptors were obtained by catalysis by GnT-III as described previously [17]. These three acceptor substrates, GnGn-bi-PA, Gn-bisected bi-PA, Glc-bisected bi-PA, were eluted at 13 min, 26 min, and 7.5 min, respectively, on a reversed phase HPLC, under the conditions described in Section 2 (Fig. 1A). When purified GnT-V was incubated with GnGn-bi-PA, Gn-bisected bi-PA, or Glc-bisected bi-PA (10 μM each) in the presence of 4 mM UDP-GlcNAc, the peaks corresponding to the reaction products were detected at 8.6 min, 18 min, and 6.0 min, respectively (Fig. 1B–D). Thus, by use of a high concentration of GnT-V (40 ng/μl), it is indicated that GnT-V is also able to transfer GlcNAc to these bisected acceptors, even if to a much lesser extent.

To confirm the transfer of a GlcNAc to the bisected oligosaccharides by GnT-V, the reaction products were collected and analyzed by ESI-MS. As shown in Fig. 2A, the *m/z* value of $(M+H)^+$ of the reaction product using GnGn-bi-PA as a substrate was 1598.5, corresponding to that of tri′-PA (Scheme 1). Similarly, the *m/z* values of the material in the product peaks using Gn-bisected bi-PA and Glc-bisected bi-PA as a substrates were 1801.5 (Fig. 2B) and 1760.5 (Fig. 2C), corresponding to those of Gn-bisected tri′-PA and Glc-bisected tri′-PA, respectively. These results verify that GnT-V is capable of transferring a GlcNAc residue to bisected acceptors.



Scheme 1. The reactions catalyzed by GnT-V. The transfer reaction of a β1,6-branched GlcNAc residue to various acceptor oligosaccharides. R represents 4GlcNAcβ1-4GlcNAc.

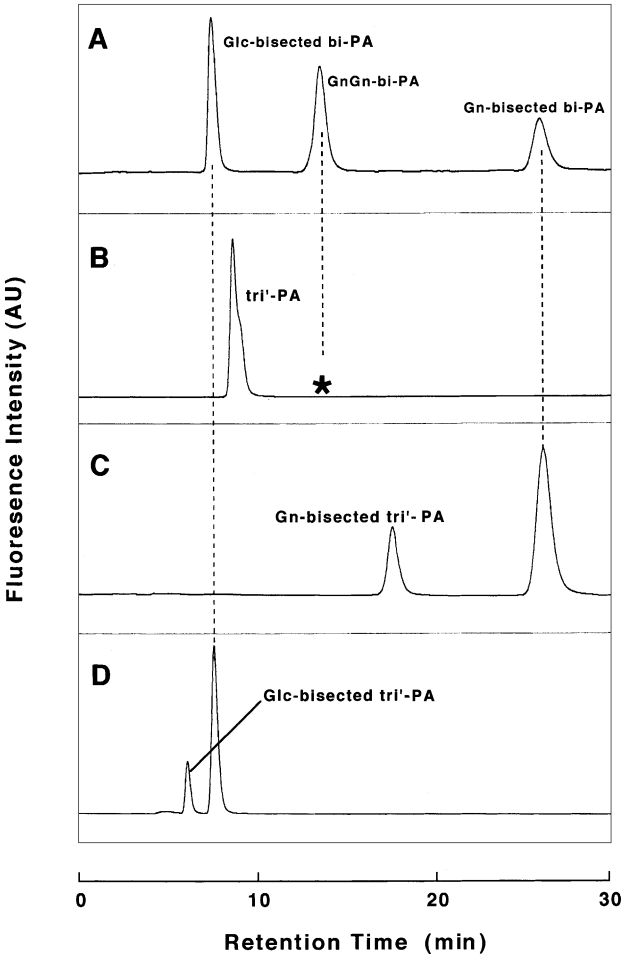


Fig. 1. Detection of GnT-V activities using various bisected oligosaccharides. The purified GnT-V was incubated with 10 μM of GnGn-bi-PA (B), Gn-bisected bi-PA (C), and Glc-bisected bi-PA (D) in the presence of 4 mM UDP-GlcNAc. The elution times for the non-reacted acceptors are shown in A. No substrate remained in the case of GnGn-bi-PA (*) because of its much faster reaction rate.

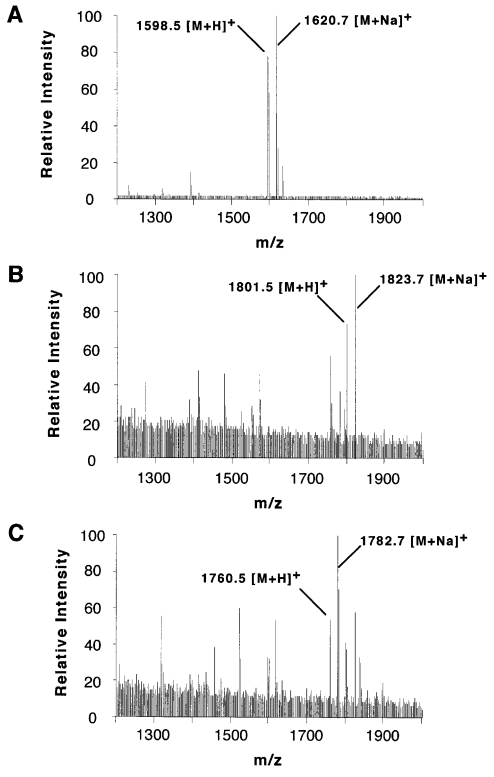


Fig. 2. ESI-MS analysis of the reaction products by GnT-V. MS spectra of the reaction products from Gn-bisected bi-PA (B) and Glc-bisected bi-PA (C) are shown with that of the product from GnGn-bi-PA (A). Detailed conditions for the analysis are described in Section 2.

The elucidation of a kinetic factor associated with the transfer rates would be important for understanding the mechanism underlying the substrate specificity of GnT-V. To address this issue, kinetic analyses for bisected acceptors as well as a non-bisected acceptor were carried out. When GnT-V activities were assayed using various concentrations of UDP-GlcNAc in the presence of a fixed concentration (10 μM) of various acceptors, it was found that the reactions followed typical Michaelis–Menten type kinetics (Fig. 3A). The apparent K_m values for the donor were 10 mM and 7.4 mM in the case where Gn-bisected bi-PA and Glc-bisected bi-PA were used as acceptors, respectively, which are slightly higher than that for GnGn-bi-PA (6.0 mM). This result indicates that the presence of a bisecting monosaccharide moiety in acceptors has no significant effect on the binding to the donor.

In order to determine the K_m values for acceptors, activities were next assayed using various concentrations of acceptors in the presence of 80 mM UDP-GlcNAc (Fig. 3B). Because this donor concentration is sufficiently high as compared to the K_m values, kinetic parameters close to the true values would be obtained for the acceptors. With respect to GnGn-bi-PA, the values for K_m (180 μM) and V_{max} (17 μmol/h/mg) are nearly the same as those reported in our previous study [16]. As summarized in Table 1, it was found that the K_m values for the three acceptors examined in this study were essentially equal. An earlier study showed that the K_m value for a trisaccharide acceptor is similar to the K_i value for its 6′-deoxy analog [22], thus permitting an assumption that K_m

Table 1
Kinetic parameters for the transfer reactions catalyzed by purified GnT-V

Acceptor substrate	Acceptor					Donor
	V_{max}		K_m	V_{max}/K_m		app. K_m
	(nmol/h/mg)	(%)	(μM)		(%)	(mM)
GnGn-bi-PA	17000	(100)	180	94	(100)	6.0
Gn-bisected bi-PA	0.76	(0.0045)	140	0.0054	(0.0057)	10
Glc-bisected bi-PA	4.32	(0.025)	82	0.054	(0.056)	7.4

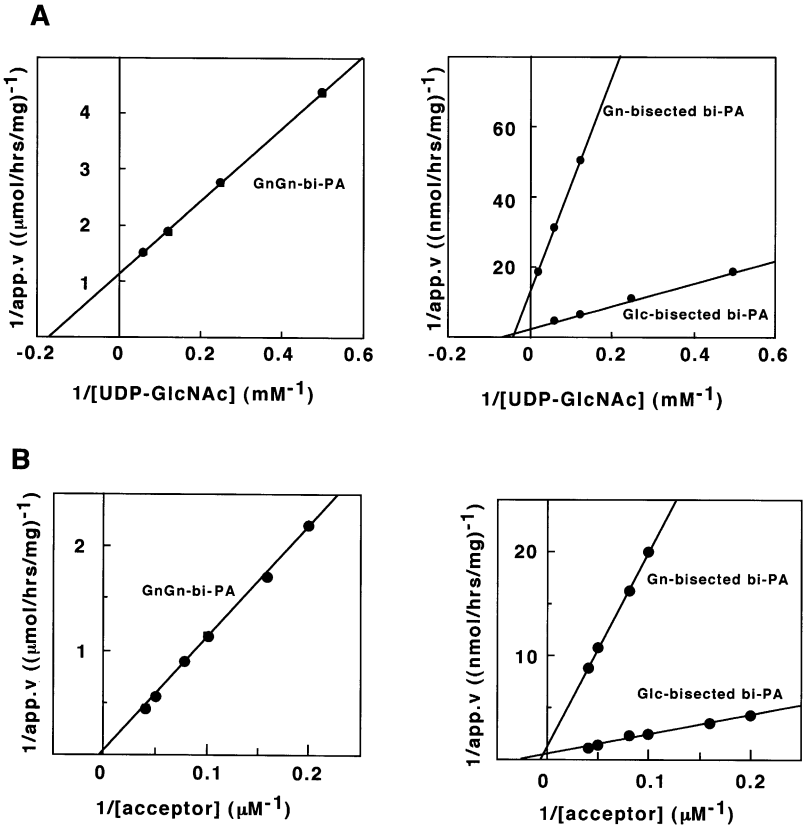


Fig. 3. Kinetic analyses of GnT-V reactions using non-bisected and bisected acceptor oligosaccharides. A: GnT-V activity was measured with various concentrations of UDP-GlcNAc in the presence of a fixed concentration (10 μM) of acceptors. The results are shown in the form of double reciprocal plots. The plots obtained using GnGn-bi-PA as an acceptor (left panel) are separated from the other two plots (right panel), because of the much faster reaction rate. B: GnT-V activity was measured with various concentrations of acceptors in the presence of a fixed concentration (80 mM) of UDP-GlcNAc. The results are shown in the form of double reciprocal plots. The plots obtained using GnGn-bi-PA as an acceptor (left panel) are separated from the other two plots (right panel), because of its much faster reaction rate.

is equal to K_d. Therefore, the results suggest that the GnT-V:donor complex, which is initially formed [23], binds to bisected acceptors comparably as to a non-bisected acceptor. This was an unexpected result because it has been thought that the inhibitory effect of the bisecting GlcNAc on the action of GnT-V is due to the induction of conformational change in the trisaccharide moiety which is recognized by the enzyme β-D-GlcNAc-(1-2)-α-D-Man-(1-6)-β-D-GlcNAc-β-OR, particularly α1,6-linked Man residue, as suggested by several reports including NMR studies [13–15,24,25].

On the other hand, the V_{max} values for Gn-bisected and Glc-bisected acceptors were 22 000 and 3900 times lower, respectively, than that for the non-bisected acceptor. Assuming that the binding mode for the GlcNAc- and Glc-bisected acceptors is the same, it seems likely that the presence of the bisecting monosaccharides more significantly affects the cata-

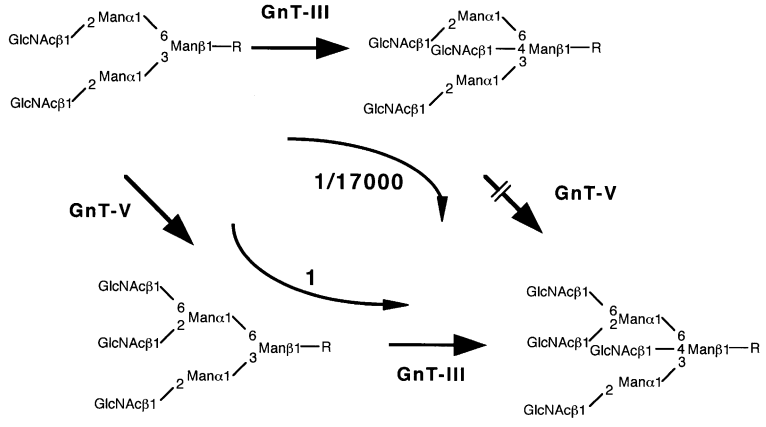


Fig. 4. Biosynthetic pathways of bisected tri′-antennary oligosaccharide. The pathways from biantennary oligosaccharide to bisected tri′-antennary oligosaccharide are shown. The double line indicates the step that is inhibited by the presence of the bisecting GlcNAc. R represents 4GlcNAcβ1-4GlcNAcβ-Asn (in polypeptide).

lytic step than the binding step. On the basis of the catalytic efficiency (V_{max}/K_m for the acceptor), the specificity of GnT-V with respect to the non-bisected acceptor was estimated to be at least 17 000 and 1700 times higher than that for Gn-bisected and Glc-bisected acceptors, respectively. This indicates that the specificity for Glc-bisected bi-PA was approximately 10 times higher, compared to the Gn-bisected bi-PA. Since the difference in the kinetic parameters between these two bisected acceptors can only be attributed to the effect of the presence of a 2-*N*-acetyl group on the bisecting monosaccharide moieties, not only the presence of a bisecting monosaccharide per se but the presence of the 2-*N*-acetyl group in the monosaccharide portion is also a significant factor that affects catalysis. In addition to possible steric hindrance, this could be rationalized, for example, by the formation of a hydrogen bond which is unfavorable for the catalysis.

The oligosaccharide moieties on glycoconjugates are assembled via the sequential action of various glycosyltransferases in the Golgi apparatus [26]. Therefore, not only the levels of expression and activities of the glycosyltransferases but the specificities toward the acceptor oligosaccharides are also critical determinants of the ultimate oligosaccharide structures. Because GnT-III and GnT-V share a common substrate, agalacto-biantennary oligosaccharide, GnT-V competes with GnT-III for a substrate (Fig. 4). As demonstrated in the present study, the V_{max}/K_m value for the Gn-bisected oligosaccharide was more than 17 000 times lower than that for the non-bisected oligosaccharide, thus, once the bisecting-GlcNAc is added the oligosaccharides would not function as an acceptor for GnT-V, in vivo. Namely, the prior action of GnT-V to GnT-III is necessary to produce the bisected tri′-antennary oligosaccharide (Fig. 4). Thus, the requirement of the sequential and ordered actions of glycosyltransferases is caused by acceptor specificity, and the 'catalysis-dependent specificity' presented in this study may be convenient for regulating the complex biosynthesis of oligosaccharide structures, in which a considerable number of glycosyltransferases are concerned.

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