Efficient Glycosynthase Mutant Derived from *Mucor hiemalis* Endo-β-N-acetylglucosaminidase Capable of Transferring Oligosaccharide from Both Sugar Oxazoline and Natural N-Glycan

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Endo-M, an endo-β-N-acetylglucosaminidase from *Mucor hiemalis*, is a family 85 glycoside hydrolase. This enzyme is unique in that it can transfer en bloc the oligosaccharide of various types of N-glycans onto different acceptors, and thereby it enzymatically generates diverse glycoconjugates. In this study, we performed mutational and kinetic studies focusing on a key catalytic asparagine 175 of Endo-M. We have shown that most of the Asn-175 mutants had significantly diminished hydrolysis activity but acted as glycosynthases capable of using synthetic sugar oxazoline for transglycosylation. Our results confirm the critical role of this asparagine residue in promoting the formation of an oxazolinium ion intermediate in the first step of the substrate-assisted catalysis. Interestingly, the N175Q mutant was found to possess dramatically enhanced glycosynthase-like activity with sugar oxazoline in comparison with N175A and a transglycosidase-like activity with “natural” N-glycan as well. These results also implicated the significance of amide side chain in the asparagine 175 of Endo-M for promoting oxazoline transglycosylation in the second step of the catalysis. The highly efficient syntheses of glycopeptides/glycoproteins by N175Q combined with synthetic sugar oxazolines or natural N-glycan substrates were exemplified. In addition, we also identified several previously unknown residues that seem to play a role in the catalysis of Endo-M.

Endo-β-N-acetylglucosaminidase (ENGase) is a glycoside hydrolase that acts on the β1,4-glycosidic linkage between the N,N'-diacetylchitobiose core of N-glycans. This type of enzymes is classified into two glycoside hydrolase (GH) families, GH18 and GH85, in the CAZY data base. GH18 includes bacterial endo-β-N-acetylglucosaminidasens, such as Endo-H (1), Endo-F1 (2), Endo-F2, and Endo-F3 (3) as well as many chitinases. On the other hand, GH85 is exclusively composed of endo-β-N-acetylglucosaminidasens from both prokaryotes and eukaryotes, such as Endo-M from *Mucor hiemalis* (4–7), Endo-A from *Arthrobacter protophormiae* (8), Endo-D from *Streptococcus pneumoniae* (9), Endo-CE from *Caenorhabditis elegans* (10, 11), and ENGase from human (12). Notably, unlike GH18 enzymes, most of the GH85 enzymes possess transglycosylation activity, i.e. the ability to transfer oligosaccharide en bloc from a donor substrate to a GlcNAc-containing backbone as an acceptor to form new glycoconjugates (13–18). This enzymatic method is one of the most promising approaches to synthesize homogeneous glycoproteins (19).

Structural and mechanistic studies with some GH18 ENGases and GH18 chitinases (20) and GH20 β-N-acetylhexosaminidasens (21) have indicated that the catalysis of these GH18 and GH20 enzymes proceeds in a substrate-assisted mechanism involving the participation of the 2-acetamide group in the substrate. In this mechanism (Scheme 1), a catalytic residue (Asp or Glu) acts as a general acid to protonate the glycosidic oxygen. Upon activation of the glycosidic bond, the 2-acetamide group of the (−1)GlcNAc acts as a nucleophile to substitute the leaving group at the anomeric center, resulting in the formation of an oxazolium ion intermediate. The catalytic carboxylate residue then acts as a general base to activate a water molecule at the catalytic center to facilitate the hydrolysis of the oxazolium ion intermediate to form the hydrolytic product. The double sn-2 type displacement results in a retaining of the anomeric configuration. In addition, several structural analyses on GH18 chitinases and GH20 β-N-acetylhexosaminidasens revealed that the proper orientation of the acetamide group may be aided by another key carboxylate residue (usually Asp) located at 1 or 2 amino acid residues upstream from the general acid/base catalytic residue (Scheme 1). The GH85 ENGases, most of which show transglycosylation

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§ The abbreviations used are: ENGase, endo-β-N-acetylglucosaminidase; CT, complex type; Endo-M, endo-β-N-acetylglucosaminidase from *M. hiemalis*; GH, glycoside hydrolase; pNP, para-nitrophenyl; 4MU, 4-methylumbelliferyl; RP-HPLC, reversed phase high performance liquid chromatography; ESI-MS, electrospray ionization-mass spectrometry; NAG-thiazoline (1,2-dideoxy-2′-methyl-α-D-glucopyranosyl[2,1-d]-Δ2-thiazoline).
activity, have been implicated to follow a similar substrate-assisted mechanism. This notion was reinforced by the fact that Endo-A and Endo-M are able to take synthetic sugar oxazolines as donor substrates for transglycosylation (22, 23). However, in contrast to GH18 ENGases, there is no second conserved carboxylate residue near the general acid/base carboxylate residue. Instead, sequence alignment has indicated the presence of a conserved Asn at the second position upstream from the general acid/base catalytic residue, which might play a similar role as the second carboxylate in the GH18 and GH20 enzymes. We have recently shown that mutation of this conserved Asn residue (Asn-175) in Endo-M resulted in almost complete loss of the hydrolytic activity of the enzyme (7), suggesting a critical role of this residue in the enzymatic catalysis. Interestingly, the N175A mutant was still able to catalyze the second step of the reaction, i.e., the mutant could use a pre-synthesized sugar oxazoline as a donor substrate for transglycosylation to form a glycopeptide but lacked the hydrolysis activity for the transglycosylation product (7). The corresponding Endo-A mutant, EndoA-N171A, was also capable of catalyzing the transglycosylation using high mannose-type glycans oxazoline as the donor substrate (24). More recently, the structures of two GH85 ENGases, Endo-A (25, 26) and Endo-D (27), were solved by x-ray crystallographic studies, which clearly confirmed the role of the conserved Asn residue at the catalytic site in orientating the 2-acetamide group and/or stabilizing the oxazolinium ion intermediate thus formed.

The novel glycosynthase, EndoM-N175A, that we have previously created has demonstrated a great potential for the synthesis of homogeneous glycoproteins carrying natural complex-type and high mannose-type N-glycans (7, 24). Despite the lack of the product hydrolysis, however, the specific activity of the glycosynthase for transglycosylation was much lower than that of the wild type Endo-M. The enzymatic reactions catalyzed by the EndoM-N175A mutant are usually slow, requiring relatively large amounts of the mutant enzyme and/or extended incubation time. To improve the catalytic efficiency of the glycosynthase for practical use, we have performed a systematic mutagenesis at the critical Asn-175 site of Endo-M, as well as site-directed mutations at other conserved Glu and Asp residues to probe whether those carboxylate residues are critical in the catalysis. The mutagenesis and subsequent enzymatic evaluation have led to the identification of an array of glycosynthase mutants that showed enhanced catalytic activity. In particular, the N175Q mutant was found to possess significantly enhanced transglycosylation activity for activated sugar oxazolines, whereas its hydrolysis activity for the product was diminished. Surprisingly, this mutant was also capable of efficiently transglycosylating N-glycan while having a significantly diminished product hydrolysis activity behaving as a typical “transglycosidase.” Furthermore, a novel mutant D279A, with a single mutation at the conserved Asp-279 residue, was also found to behave as a glycosynthase. Kinetic studies reveal the nature of the enhancement of the transglycosylation efficiency and provide further insights into the transglycosylation mechanism of Endo-M.

EXPERIMENTAL PROCEDURES

Materials—The biantennary complex-type sialylglycopeptide (SGP), Lys-Val-Ala-Asn((NeuAc-Gal-GlcNAc-Man)$_n$(GlcNAc-$n$)-Lys-Thr, the biantennary complex-type sialylglyco-dansylated asparagine (dansylated SGN), and the Man$_n$GlcNAc$_r$-Asn were prepared according to reported procedures (7); the high mannose-type oxazoline (Man$_n$GlcNAc$_r$-oxazoline) and the complex-type oxazoline (CT-oxazoline) were synthesized as described previously (7, 24); the GlcNAcpentapentapeptide (Glu-Asn(GlcNAc)-Ile-Thr-Val) derived from erythropoietin and GlcNAc-DS2(Gly-Gln-Asn(GlcNAc)-Asp-Thr-Ser-Gln-Thr-Ser-Pro-Ser) were synthesized as we described previously (7, 28). $p$-Nitrophenyl-$eta$-d-N-acetylglucosaminide (pNP-GlcNAc) was purchased from Nacalai Tesque (Japan); 4-methylumbelliferyl-$eta$-d-N-acetylglucosaminide (4MU-GlcNAc) and chitinase from Streptomyces griseus were purchased from Sigma.

Methods—Analytical reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a HITACHI D-2000 Elite instrument with a Cosmosil 5C18-AR II column (4.6 × 150 mm, Nacalai Tesque, Japan) at 40 °C or on a Waters 626 HPLC instrument with a Symmetry300$^\text{TM}$ C18 column (5.0 μm, 4.6 × 250 mm) at 40 °C. The column was eluted with a linear gradient using one of the following four methods, depending on the properties of the compounds to be separated: method A, 0–15% MeCN containing 0.1% trifluoroacetic acid for 12 min; method B, 22% MeCN containing 25 mM sodium borate (pH 7.5) for 15 min and 22–35% for next 11 min; method C, 5–20% MeCN containing 0.1% trifluoroacetic acid for 20 min; and method D, 0–30% MeCN containing 0.1% trifluoroacetic acid for 20 min.

Site-directed Mutagenesis and Expression and Purification of Recombinant Endo-M in Escherichia coli—The mutants were generated by PCR using KOD-plus DNA polymerase (Toyobo, Japan) according to the procedure for a QuiKChange site-directed mutagenesis kit (Strategene) using the pET23b-Endo-M plasmid, containing cDNA fragment of the wild type Endo-M fused a His$_8$ tag at C terminus, as a template DNA (7). For saturation mutagenesis of Asn-175 residue, a forward primer, 5′-CTATGGTTTTTGGATGGCTGGTTGTNNSATTGA-ATGCGAATTC-3′, and a reverse primer, 5′-GAAATTCGCG-ATTCAATSNNGAACCAACCAGGCCATCABAAAACCATAG-3′, were used. Mutations were confirmed by DNA sequencing. The plasmids containing mutated Endo-M genes were introduced into E. coli BL21 (DE3). Expression and purification of
enzymes were carried out using the Ni\(^{2+}\)-charged chelating column as described in our previous paper (7). The protein concentrations were quantified using the BCA protein assay kit (Thermo) and used in the following enzyme assay.

**Enzyme Assay**—Assay for the hydrolysis activity of each enzyme was performed at 30 °C with 4 mM SGp or 0.31–20 mM DNS-SGN as a substrate in 50 mM sodium phosphate buffer (pH 6.5, 80 µl), and the reaction was stopped by boiling for 3 min. The reaction mixtures, including SGP or dansylated SGN, were analyzed by RP-HPLC using method A or method B, respectively. Residual substrates and the hydrolysis products in the reaction mixture were detected at UV 214 nm or fluorescence (excitation, 355 nm; emission, 460 nm) for SGP or DNS-SGN, respectively. One unit of the enzyme was defined as the amount of protein yielding 1 µmol of peptide-GlcNAc per min at 30 °C by using SGP as a donor substrate.

The transglycosylation reaction with synthetic sugar oxazoline was assayed as follows: 7.5 mM Man\(_9\)GlcNAc-oxazoline, 20 mM \(p\)-NP-GlcNAc, and 0.4 µg of the wild type (WT) Endo-M or 4.0 µg of each mutant enzyme was incubated at 30 °C in a sodium phosphate buffer (50 mM, pH 6.5, 80 µl) containing 10% DMSO, which enhances the solubility of the \(p\)-NP-GlcNAc in the buffer. The reaction mixture was analyzed by RP-HPLC using method C. The residual acceptor substrate (\(p\)-NP-GlcNAc) and the transglycosylation product (Man\(_9\)GlcNAc\(_2\)pNP) were detected at 280 nm with a UV detector. The quantity of SGP was measured using a chitinase-coupled method described previously (7). This method was able to monitor the transglycosylation product once formed without the enzymatic re-hydrolysis, because co-incubated chitinase promptly hydrolyzes the transglycosylation product to yield a fluorescence as follows: the solution of 1 mM SGP, 2.5 mM 4MU-GlcNAc, and 100 milliunits of chitinase from S. griseus was incubated with WT (3 µg), Y217F (3 µg), or N175Q (15 µg) in 50 mM sodium phosphate (pH 6.5) containing 10% DMSO. The transglycosylation reactions were monitored as described previously (7).

TLC analysis of the transglycosylation reaction was carried out as follows: 1 mM SGP, 10 mM GlcNAc, and each enzyme, WT (0.56 µg), Y217F (0.80 µg), or N175Q (8.0 µg) were incubated in 25 mM ammonium acetate (pH 6.0, 80 µl) at 30 °C. Sugars were detected in the same manner as described previously (7).

Transglycosylation to GlcNAc-pentapeptide using SGP as the donor substrate was carried out as follows: 9 mM SGP, 3 mM GlcNAc-pentapeptide and WT (1 µg), or Y217F (1.2 µg) or N175Q (12 µg) were incubated in 50 mM sodium phosphate (pH 6.5, 100 µl) at 30 °C. Aliquots (10 µl each) were taken at intervals and heated at 100 °C for 3 min to stop the reaction. The transglycosylation product (NeuAc-Gal-GlcNAc-Man\(_2\)Man-GlcNAc\(_2\)-pentapeptide) and the residual SGP were quantified using RP-HPLC in method D at UV 214 nm in a same manner as described previously (7).

**Kinetic Studies Using High Mannose-type Oxazoline as Donor Substrate**—The transglycosylation reactions for kinetic studies of the mutants were assessed at 30 °C for 5 or 10 min in a reaction buffer containing 100 mM phosphate buffer (pH 7.2), 10 mM Man\(_9\)GlcNAc-oxazoline, and an appropriate amount of respective enzyme (0.25 µg of N175Q, 0.5 µg of Y217F/N175Q, 0.5 µg of N175G, 0.5 µg of D279A, or 6 µg of N171A) in a total of 5 µl. To determine the apparent \(K_m\) values for the acceptor substrate, the concentration of Man\(_9\)GlcNAc-oxazoline was fixed at 10 mM, whereas the concentration of the acceptor GlcNAc-pNP was varied as 0.63–20 mM. To determine the \(V_m\) values for Man\(_9\)GlcNAc-oxazoline, the acceptor concentration was fixed at 20 mM, and the concentration of Man\(_9\)GlcNAc-oxazoline was varied as 0.63–10 mM. The reaction was monitored by RP-HPLC using method C. The hydrolysis of Man\(_9\)GlcNAc-oxazoline by Endo-M and its mutants was performed at 30 °C in a phosphate buffer (pH 7.2) containing 10% DMSO. A solution of Man\(_9\)GlcNAc-oxazoline at various concentrations (0.31–10 mM) in a phosphate buffer (200 mM, pH 7.2, containing 10% DMSO, total volume, 5 µl) was incubated with 0.3 µg of WT or 0.4 µg of N175Q or 4 µg of N175A at 30 °C. The reaction was terminated after 5 min by the addition of an equal volume of 10% tetrahydrofuran in aqueous sodium hydroxide (0.2 M). The aliquots were then analyzed by high performance anion-exchange chromatography coupled with pulsed amperometric detection, and the hydrolytic product Man\(_9\)GlcNAc was quantified according to our previously described method (30). The parameters \(K_m\) and \(V_max\) were obtained by fitting the experimental data into the Michaelis-Menten kinetics model in GraphPad Prism.

**Enzymatic Synthesis of High Mannose-type and Complex-type Glycoforms of CD52**—A solution of GlcNAc-CD52 (7 mM) and Man\(_9\)GlcNAc-oxazoline (35 mM) or CT-GlcNAc-oxazoline (35 mM) in a phosphate buffer (50 mM, pH 7.0, 30 µl) was incubated with N175Q (20 µg) at 30 °C. After 10 min, the transglycosylation was completed to give the corresponding glycoform of CD52. The transglycosylation products were isolated by reverse-phase HPLC and characterized by ESI-MS. For analytical HPLC, Symmetry300™ C18 column (5 µm, 4.6 × 250 mm) at 40 °C, a linear gradient of 0–10% MeCN containing 0.1% trifluoroacetic acid in 20 min at the flow rate of 1 ml/min was used. For high mannose-type glycoform Man\(_9\)GlcNAc\(_2\)-CD52 was an 84% yield and \(t_{1/2} = 12.6\) min (analytical HPLC); ESI-MS was as follows: calculated for C\(_{113}\)H\(_{190}\)N\(_{18}\)O\(_{78}\), M = 3071.14; found, 3071.14 [M + H]\(^+\). For complex-type glycoform CT-GlcNAc\(_2\)-CD52 was a 76% yield and \(t_{1/2} = 12.5\) min (analytical HPLC); ESI-MS was as follows: calculated for C\(_{107}\)H\(_{176}\)N\(_{20}\)O\(_{69}\), M = 2829.09; found, 1415.68 [M + 2H]\(^2+\), 944.09 [M + 3H]\(^3+\), 708.33 [M + 4H]\(^4+\).

**RESULTS**

Site-directed Saturation Mutagenesis at Asn-175—We previously found that the N175A mutant of Endo-M behaves as a “glycosynthase”; it can use the activated sugar oxazoline as a donor substrate for transglycosylation, but it lacks the activity to hydrolyze the product formed (7). Nevertheless, the specific activity of the N175A mutant was very low and therefore required a large amount of enzyme to accomplish a synthesis.
Endo-M Mutant Acts like Glycosynthase and Transglycosidase

To search for more efficient glycosynthase mutants, we tried to systematically replace Asn-175 with other 19 natural amino acid residues except proline. The resulting 18 N175 mutants were overproduced in E. coli, and their hydrolysis activities for N-glycan and transglycosylation activities of sugar oxazoline were assessed. As to the specific hydrolysis activity for N-glycopeptide SGP, all the Asn-175 mutants showed none or only marginal activity, confirming the critical role of the Asn-175 residue in N-glycan hydrolysis. Only six mutants, N175D, N175Q, N175H, N175S, N175M, and N175T, retained some residual hydrolytic activity (∼0.5% that of the wild type) (Table 1).

Next, the transglycosylation activities of the Asn-175 mutants using sugar oxazoline were compared with that of the N175A (Fig. 1A). Despite their dramatic loss of the hydrolysis activity, most of the Asn-175 mutants showed transglycosylation activity with Man$_9$GlcNAc-oxazoline (Fig. 1). Among all these glycosynthase-like mutants, N175Q showed extraordinarily high transglycosylation activity, and the initial rate was about 9 times and 1.5 times higher than those of N175A and WT, respectively (Fig. 1B). We also created the double mutant N175Q/Y217F, because the Y217F mutant was previously found to accelerate transglycosylation by increasing affinity for acceptor substrate (7). It was found that the double mutant was a glycosynthase, but unexpectedly the additional mutation at Tyr-217 did not further enhance the activity (Fig. 1, A and B). Some of the Asn-175 mutants replaced by bulky residues, such as Arg, Lys, Trp, and Tyr, did not show any detectable transglycosylation activity in our assay conditions (data not shown). The above results clearly demonstrate that the Asn-175 residue plays an important role not only in the formation and stabilization of the oxazoline but also in the second step involving the transglycosylation and hydrolysis of the oxazolinium ion intermediate.

**Mutation at Conserved Carboxylate Residues**—It was clear that Asn-175 has a key role in the formation of the oxazolinium ion intermediate. However, in GH18 and GH20 enzymes, there is a conserved aspartate residue, not asparagine residue, at the corresponding position to promote oxazolinium ion formation. Thus, it is still possible that other conserved carboxylate residues might function in concert with the conserved asparagine residue for the catalysis. To test this hypothesis, we carried out site-directed mutagenesis on other conserved carboxylate residues and examined each effect on hydrolytic activity for natural N-glycan substrate (Table 2) and transglycosylation activity for sugar oxazoline (Fig. 2). By amino acid sequence comparison with other GH85 enzymes, Asp-89, Asp-147, Asp-158, Asp-170, Glu-177, Asp-218, and Asp-279 of Endo-M were found to be highly conserved among the enzymes in the family. Each carboxylate residue was replaced with alanine, and all of the mutants except D89A were overproduced in *E. coli*. It was found that mutation at the general acid/base residue Glu-177 with alanine resulted in a complete loss of hydrolytic activity for SGP, which is consistent with our previous study implicating the critical role of the Glu-177 in the enzymatic catalysis (7). Interestingly, the D170A, D218A, and D279A mutants also demonstrated dramatically reduced hydrolysis activity, suggesting that these carboxylate residues also play an important part in the catalytic hydrolysis. In fact, recent structural analysis of GH85 homologous enzymes, Endo-A and Endo-D, implicated that these equivalent residues are in the active site of the enzyme (25–27). The D147A and D158A did not show marked reduction, indicating that these two residues are not required for hydrolytic activity.

Using Man$_9$GlcNAc-oxazoline as a donor substrate, we assessed the transglycosylation activity of these mutants and compared them with that of N175A (Fig. 2). Except for Glu-177 mutants, all the other mutants showed transglycosylation activities for sugar oxazoline. Although the N175A mutant was able to steadily catalyze the trans-

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**TABLE 1**

| Mutant | Specific hydrolysis activity$^a$ | Percentage of specific hydrolysis activity |
|--------|----------------------------------|------------------------------------------|
| Wild type | 9.5 | 100 |
| N175A | $1.1 \times 10^{-2}$ | 0.12 |
| N175G | $1.8 \times 10^{-3}$ | 0.019 |
| N175V | $7.2 \times 10^{-3}$ | 0.076 |
| N175L | $4.9 \times 10^{-3}$ | 0.002 |
| N175I | $6.0 \times 10^{-4}$ | 0.0063 |
| N175D | $4.2 \times 10^{-4}$ | 0.044 |
| N175E | $1.5 \times 10^{-4}$ | 0.016 |
| N175Q | $6.1 \times 10^{-4}$ | 0.064 |
| N175H | $5.0 \times 10^{-4}$ | 0.52 |
| N175C | $7.6 \times 10^{-4}$ | 0.080 |
| N175S | $5.5 \times 10^{-4}$ | 0.057 |
| N175M | $4.0 \times 10^{-4}$ | 0.42 |
| N175T | $4.5 \times 10^{-4}$ | 0.047 |
| N175Y | $7.8 \times 10^{-4}$ | 0.0082 |
| N175F | $1.8 \times 10^{-4}$ | 0.019 |
| N175W | $3.1 \times 10^{-4}$ | 0.0033 |
| N175R | $4.2 \times 10^{-5}$ | 0.00044 |
| N175K | $2.0 \times 10^{-4}$ | 0.0021 |

$^a$ The hydrolysis activity was determined using 4 mM SGP as the substrate.
glycosylation without hydrolyzing the product, D158A, D170A, and D218A mutants led to a gradual hydrolysis of the transglycosylation product. As expected, D147A, which did not change its hydrolytic activity, showed a similar pattern with WT in transglycosylation. Interestingly, D279A was found to catalyze transglycosylation of the sugar oxazoline without hydrolyzing the product as in the case with N175A, implicating that the D279A mutant is another glycosynthase derived from Endo-M.

In addition to E177A, we also created and examined the E177H and E177Q mutants, as Fairbanks and co-workers (31) have recently reported that the corresponding Endo-A mutants (EndoA-E173H and EndoA-E173Q) possess glycosynthase-like activity and are able to take Man₉GlcNAc-oxazoline as substrate. Significant differences were shown in the values of specific hydrolysis activity with either Man₉GlcNAc-oxazoline/Man₉GlcNAc-oxazoline, demonstrating that the Glu-177 is an essential residue for activation of an acceptor in the oxazoline transglycosylation reaction. It is clear that the function of the Glu-177 in Endo-M could not be substituted by a histidine or a glutamine residue, in contrast to the work recently reported by Fairbanks and co-workers (31) on the corresponding Endo-A mutants. Furthermore, we have found that the hydrolytic activity of EndoA-E173A mutant could be rescued by addition of exogenous azide (32), whereas the hydrolytic activities of the Endo-M mutants (E177A and E177G) could not be rescued by incorporation of an exogenous nucleophile such as azide (data not shown). One possibility is that the mutation of Glu-177 of Endo-M might cause a more unfavorable conformational change in the active site than that of Endo-A. Regardless, the structural analysis of Endo-M should be awaited to clarify these differences.

Kinetic Studies of the Selected Mutants—On the basis of the preliminary screening results, the following single mutants N175Q, N175G, D279A and a double mutant N175Q/Y217F were selected, and their superior transglycosylation activity (in comparison with the previously reported mutant N175A) toward both pNP-GlcNAc and GlcNAc-pentapeptide was confirmed in a triplicate assay (data not shown). To quantify the effect of the mutations, the kinetics for the transglycosylation reaction of these superior mutants were analyzed and compared with N175A (Table 3). As to the $K_m$ value for Man₉GlcNAc-oxazoline, there was no significant difference between three single mutants, N175A, N175G, and N175Q, indicating that these three mutations did not affect binding of the sugar oxazoline. On the other hand, the N175G and N175Q showed an ~2-fold decreased $K_m$ value for the acceptor substrate compared with N175A, indicating that the Gly and Glu mutants have a moderately enhanced affinity for the acceptor substrate. Significant differences were shown in the values of $k_{cat}$ of these three mutants. The $k_{cat}$ value of N175G was 6-fold higher than that of N175A. Consistently, mutations of Asn-175 to a nonpolar residue having a smaller side chain showed a tendency to enhance the transglycosylation activity for sugar oxazoline (Fig. 1B). Notably, the $k_{cat}$ of N175Q, which showed significantly enhanced transglycosylation activity for Man₉GlcNAc-oxazoline (Fig. 1, A and B), was about 23-fold higher than that of N175A (Table 3). Thus, in comparison with the Asn to Ala mutation, changing the Asn-175 to a similar Glu residue led to a diminished hydrolytic activity, but yet it could still maintain a high turnover rate for transglycosylation with the activated sugar oxazoline. The results suggest that the amide side chain in the Asn or Gln at the 175 position plays an

### TABLE 2
Comparison of the specific hydrolysis activities of the mutants at conserved carbohydrate residues for natural N-glycan substrate

| Mutant | Specific hydrolysis activity | Percentage of specific hydrolysis activity |
|--------|-----------------------------|------------------------------------------|
| Wild type | 9.5 | 100 |
| D147A | 7.8 | 82 |
| D158A | 1.8 | 19 |
| D170A | 1.1 x 10⁻¹ | 1.2 |
| N175A | 1.1 x 10⁻² | 0.12 |
| E177A | N.D | |
| E177Q | 3.0 x 10⁻₃ | 0.032 |
| E177H | 2.1 x 10⁻¹ | 0.0022 |
| D218A | 4.5 x 10⁻¹ | 0.47 |
| D279A | D.0 x 10⁻⁴ | 0.0063 |

* The hydrolysis activity was determined using 4 mM SGP as the substrate.

** ND means not determined.

### TABLE 3
Kinetic parameters of Endo-M mutants for transglycosylation using Man₉GlcNAc-oxazoline as a donor substrate and pNP-GlcNAc as an acceptor substrate

| Mutant | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$ | $k_{cat}/K_m$ |
|--------|---------|-------------|------|-------------|
| N175A | 11 ± 0.5 | 2.4 ± 0.37 | 47 | 4.7 |
| N175G | 64 ± 4.8 | 2.3 ± 0.47 | 27 | 27 |
| N175Q | 250 ± 24 | 2.1 ± 0.51 | 120 | 120 |
| Y217F/N175Q | 41 ± 2.5 | 0.47 ± 0.15 | 87 | 87 |
| D279A | 230 ± 48 | 5.4 ± 2.3 | 43 | 43 |
| pNP-GlcNAc | 11 ± 0.54 | 7.4 ± 0.83 | 4.7 | 4.7 |
| N175G | 66 ± 5.4 | 4.2 ± 0.97 | 16 | 16 |
| N175Q | 250 ± 17 | 3.5 ± 0.84 | 72 | 72 |
| Y217F/N175Q | 67 ± 4.4 | 9.1 ± 1.3 | 7.3 | 7.3 |
| D279A | 150 ± 15 | 2.0 ± 0.76 | 77 | 77 |

* The $k_{cat}$ and $K_m$ values represent mean ± S.D. (n = 3).
important role in promoting the transglycosylation with the sugar oxazoline.

We have previously shown that Y217F mutant enhanced the transglycosylation activity (7). This could be caused by exclusion of water in the active center and/or enhanced affinity for acceptor substrate. However, the net result is that the double mutant N175Q/Y217F neither increased the overall catalytic efficiency nor enhanced the transglycosylation activity in comparison with the single mutant N175Q (Fig. 1, A and B). Our kinetic data showed that N175Q/Y217F had a decreased affinity for pNP-GlcNAc ($K_m = 9.1$ mM) in comparison with N175Q (3.5 mM). This result seems to be in contrast to our previous observation with Y217F single mutant. More surprisingly, its affinity for Man$_n$GlcNAc-oxazoline has increased significantly ($K_m = 0.47$ mM for the double mutant and $K_m = 2.1$ mM for N175Q). These results implicate that the double mutation might cause changes of the local structure at the catalytic region, making the analysis of the kinetic data complicated.

Despite its dramatically diminished hydrolysis activity toward N-glycan SGP (Table 1 and Fig. 2), the D279A mutant was found to be able to catalyze transglycosylation with sugar oxazoline, implicating that Asp-279 may also take an important part in the first step of the catalysis for supporting the catalytic Glu-177 and key Asn-175. Notably, the $k_{cat}$ of D279A for the transglycosylation of Man$_n$GlcNAc-oxazoline is over 20-fold higher than that of N175A, except that its affinity for the Man$_n$GlcNAc-oxazoline, as measured by the $K_m$ data, was about 2-fold lower than that of N175A (Table 3). On the other hand, the $k_{cat}$ of D279A for the acceptor pNP-GlcNAc was also much higher (15-fold) than that of N175A, whereas its affinity to the acceptor was also increased (Table 3). These data indicate that the D279A is yet another glycosynthase superior to the first glycosynthase N175A and implicate that the Asp-279 residue may also play an important role in the first step in the catalysis facilitating the formation and/or stabilization of the oxazolinium ion intermediate. Structural analysis of Endo-D revealed that Glu-441 of Endo-D, which is corresponding to Asp-279 of Endo-M, is hydrogen bonding with O6 of NAG-thiazoline (1,2-dideoxy-2'-methyl-α-D-glucopyranosyl-2,1-d-thiazoline) (27). These results suggest that Asp-279 of Endo-M may be important for facilitating oxazoline formation in the first step. Thus, mutation of this residue resulted in the cessation of product hydrolysis activity, but its ability to accommodate pre-formed sugar oxazoline is still maintained for transglycosylation.

Our initial screening of the glycosynthase mutants for transglycosylation activity identified N175Q as the most effective mutant for transglycosylation with minimal hydrolytic activity for the product. Nevertheless, we observed that N175Q also possesses significant residual activity to hydrolyze the activated sugar oxazoline. For comparison, we have performed kinetic studies on the hydrolysis of Man$_n$GlcNAc-oxazoline by N175Q, N175A, and WT in the absence of an acceptor. Although N175Q showed much lower activity to hydrolyze the activated donor substrate than WT, it does hydrolyze the substrate much faster than N175A. These three mutants showed similar $K_m$ values, but the $k_{cat}$ values varied significantly. The $k_{cat}/K_m$ value of WT is 570 min$^{-1}$ mM$^{-1}$, about 3.5-fold higher than that of N175Q (170 min$^{-1}$ mM$^{-1}$). The $k_{cat}/K_m$ value of N175A (11 min$^{-1}$ mM$^{-1}$) was about 16-fold lower than that of N175Q. Nevertheless, the remarkably enhanced transglycosylation activity (Table 3) together with its reduced product hydrolysis activity makes N175Q a valuable mutant for glycoconjugate synthesis.

**Exemplary Synthesis of Defined Glycoforms of Sperm Antigen CD52 Using N175Q with Sugar Oxazoline as the Donor Substrate**—To demonstrate the usefulness of N175Q with sugar oxazoline for the synthesis of complex N-glycopeptides, two glycoforms of the sperm antigen CD52 were synthesized using N175Q as the enzyme with the corresponding high mannose-type oxazoline (Man$_n$GlcNAc-oxazoline) or the complex-type oxazoline (CT-oxazoline) (Scheme 2). CD52 is a glycosylphosphatidylinositol-anchored cell surface antigen expressed on almost all human lymphocytes and sperm cells. It is one of the smallest natural glycoproteins consisting of only 12 amino acid residues but carries a large complex-type N-glycan at the Asn-3 residue. It was observed that the transglycosylation reaction proceeded quickly (less than 10 min) to give the corresponding CD52 glycoforms in a high yield (~80%, Fig. 3, A and B). The products were easily isolated by HPLC and characterized by ESI-MS. In comparison, the reaction with the same amount of N175A for 60 min gave only less than 20% yield of the respective transglycosylation product (Fig. 3, A and B), although prolonged reaction time and a larger amount of N175A could achieve a higher yield of transglycosylation. These data clearly show the enhanced efficiency of the new glycosynthase mutant for synthetic purpose than the first generation glycosynthase mutant N175A. It should be mentioned that we have previously synthesized several glycoforms of CD52 using wild type Endo-M, but the yield was less than 10% for the complex-type glycoform (28).

**Activity of N175Q for Transglycosylation with Natural N-Glycopeptide SGP**—We noticed that all of the Asn-175 mutants showed significantly decreased hydrolytic activity for natural N-glycan SGP, but some of them, replaced by residues having reactive group including Asp, Gln, His, Ser, and Thr, still retained marginal transglycosylation activity for the ground-state natural N-glycans such as SGP (data not shown). Considering industry application of the enzyme for glycoconjugate syntheses, efficient syntheses by using natural ground-state glycopeptides like SGP will be in more demand because such natural substrates are easily available in large amounts and more stable than synthetic sugar oxazolines. Therefore, we also examined the efficacy of N175Q for transglycosylation using natural N-glycan SGP as a donor in comparison with Y217F, which we previously found as the most promising mutant of Endo-M for transglycosylation using natural N-glycans. At first, we monitored the transglycosylation reaction using the chitinase-coupled method as described previously (29). This assay can monitor the amount of the transglycosylation product once formed in situ without the reduction caused by enzymatic re-hydrolysis. As a result, Y217F gave a 6-fold larger amount of the transglycosylation product than WT and reached plateau after 80 min because of the substrate depletion (Fig. 4A). Surprisingly, the transglycosylation of N175Q increased continuously up to 120
min, and the amount of the product was more than 12-fold of WT (2-fold of Y217F). We then analyzed the transglycosylation and hydrolysis by conventional TLC method using equal catalytic units of the enzyme with SGP as a donor and GlcNAc as an acceptor (Fig. 4B). Consistently, Y217F clearly gave a larger amount of the transglycosylation product and a smaller hydrolysis product than the WT. As to N175Q, although the reaction rate was relatively low, the ratio of transglycosylation/hydrolysis was seemingly higher than that of Y217F. Notably, the product hydrolysis was much more suppressed in N175Q than in Y217F, implicating N175Q behaves like a transglycosidase for natural donor substrates.

Next, we further evaluated the efficacy of the “transglycosidase-like” activity of N175Q with natural N-glycan as the donor substrate in glycopeptide synthesis. A GlcNAc-containing pentapeptide and SGP were used as an acceptor and a donor, respectively, and the products were quantified by RP-HPLC (Scheme 3 and Fig.
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As a result, N175Q gave a higher yield of the transglycosylation product and a much lower hydrolysis of the starting material than those catalyzed by Y217F. These results indicate that N175Q is a mutant superior to WT and Y217F for transglycosylating natural N-glycans. In comparison, the $K_m$ value of N175Q for the acceptor GlcNAc-pentapeptide was about 3 mM, whereas that of WT and Y217F were 10 and 2 mM, respectively, indicating that the affinity of the N175Q for acceptor was higher than the WT but moderately lower than Y217F. The $k_{cat}/K_m$ value of N175Q (0.58 x 10^-3 mm^-1 s^-1) for transglycosylation was ~4-fold smaller than that of Y217F (0.23 x 10^-2 mm^-1 s^-1). Nevertheless, significant differences were shown in kinetic parameters of N175Q for the hydrolysis of N-glycan. The parameters were examined using dansylated SGN (biantennary complex-type sialylglycosaminoglycan) as a substrate. Consistent with our previous study, the $k_{cat}/K_m$ value of Y217F (0.59 mm^-1 s^-1) was moderately decreased to about half that of WT (1.5 mm^-1 s^-1). It was found that the $k_{cat}/K_m$ value of N175Q (0.51 x 10^-2) was ~100-fold smaller than that of Y217F, because of its significantly high $K_m$ value ($K_m$ >33 mM). The moderately reduced $k_{cat}/K_m$ of N175Q for transglycosylation of N-glycan, coupled with its remarkably decreased $k_{cat}/K_m$ for product hydrolysis, resulted in a steady transglycosylation and continuous accumulation of the transglycosylation product.

**DISCUSSION**

We have previously found that the N175A mutant of Endo-M exhibited glycosynthase-like activity using sugar oxazoline as a donor substrate, suggesting the critical participation of Asn-175 residue in the formation of an oxazoline intermediate, the first step of substrate-assisted catalysis. In this study, we carried out mutational and kinetic studies focusing on the Asn-175 of Endo-M. It was found that the N175Q mutant demonstrates noticeable glycosynthase-like activity for synthetetic sugar oxazoline and transglycosidase-like activity for natural N-glycan. These results implicate the significance of the amide group in the side chain at this position in both promoting the sugar oxazolinium ion intermediate formation (in the first step) and the subsequent transglycosylation with sugar oxazoline (the second step in catalysis). Additionally, we revealed several other conserved residues that are involved in catalysis of Endo-M.

Recent structural analyses of GH85 enzymes, Endo-A (25, 26) and Endo-D (27), strongly support the assumption that the conserved asparagine residue (Asn-171 in Endo-A and Asn-335 in Endo-D) of the GH85 enzymes takes over the key aspartate residue of GH18 and GH20 enzymes in orientating the 2-acetamide group of GlcNAc in the substrates to form oxazolinium ion intermediate (Scheme 1). Our experimental data showed that any mutation at Asn-175 in Endo-M diminished the hydrolytic activity for N-glycan substrates (Table 1), confirming that Asn-175 is essential for the formation of oxazoline ring, the first step of the catalysis. Even in the N175Q mutant, the hydrolytic activity for...
N-glycan was very low. Kinetic studies showed that the $K_m$ value of N175Q for N-glycan in hydrolysis was indeed extraordinarily high. These results suggest that the extension of the side chain of this residue (Asn to Gln) by one carbon might disturb the hydrogen bonding between the amide group of this residue and the acetamide of $\text{(-1)GlcNAc}$ in the substrate. This characteristic is sufficient for preventing quick re-hydrolysis of transglycosylation product once formed and may also allow its much faster release.

On the other hand, our results indicated that Asn-175 is not essential for the second-step reaction, as most of the Asn-175 mutants still retained the ability to process oxazoline substrates in transglycosylation reactions (Fig. 1A). Nevertheless, it was also indicated that mutations at this critical position to different residues significantly changed its turnover rate of sugar oxazoline (Fig. 1B and Table 3). The structural analysis of Endo-D has revealed a hydrogen bonding between the conserved general acid/base residue Glu-337 and the Asn-335 residue (27), suggesting that this Asn might act together with Glu-337 to aid the oxazoline processing. In the case of Endo-M, mutation of the Asn-175 to Gln significantly enhanced the transglycosylation activity of oxazoline (Fig. 1B and Table 3). These results suggest that the amide residue in the glutamine side chain might be able to play the same role as that of the Asn-175 residue in cooperating with the general acid/base residue for transglycosylation, maintaining a high turnover rate as seen in the wild type Endo-M. In other words, in comparison with N175A, the glutamine residue in N175Q could still favorably interact with the...
Glu-177 residue to promote transglycosylation of oxazoline (Fig. 5, A and B). Additionally, in Endo-A transglycosylation may require the opening of the gate for the acceptor; the distance between Trp-216 and Trp-244 (Trp-228 and Trp-251, respectively in Endo-M) becomes wider to allow passage of an acceptor into the active site (26). Asn-to-Gln replacement at the 175-residue of Endo-M might give enough time lag and/or cause more favorable conformational changes to open the gate, rather than stabilize the oxazoline intermediate. As to whether the nitrogen or the oxygen of this asparagine is oriented to accept a hydrogen bond from the substrate amide (25, 27), the former scenario that this asparagine acts as the imidic acid tautomer with the nitrogen-oriented 2-acetamide of the substrate might be more likely considering our results, because in that case, the reaction proceeds via an uncharged oxazoline intermediate, which does not need stabilization by the asparagine. Furthermore, given that the replacement of the amide group of Asn-175 by the carboxyl group (Asn to Asp or Gln to Glu) indeed significantly reduces its catalytic efficiency both for hydrolysis of N-glycan (Table 1) and oxazoline processing (Fig. 1, A and B) suggests that this Asn of GH18 enzymes might function in a way fundamentally different from Asp of GH18 and GH20 enzymes. Thus, the substituted Asn of GH185 enzymes should have a significant impact on its transglycosylation/hydrolysis activity.

Structural analyses of Endo-A and Endo-D revealed that the phenyl groups of several aromatic residues, including Tyr-205 and Tyr-373, respectively (equivalent to Tyr-217 of Endo-M), are likely to take a primary role in stabilizing the oxazoline ring in a transition state (27). Consistently, in our previous study, the mutation of Tyr-217 to residues other than phenylalanine abolished the enzymatic activity (7). A loss of the hydroxyl group may create a more hydrophobic environment to enhance the binding of the oxazoline ring, and it might cause interference in the following oxazoline processing. Given that the hydroxyl group of Tyr-205 of Endo-A is close to the oxygen atom of the C2-acetamide group (26), there is another possibility that, in the absence of Asn-175 residue, the hydroxyl group of Tyr-217 might alternatively play an important role in facilitating the subsequent transglycosylation of the sugar oxazoline.

It was suggested that hydrogen bonding between Endo-D and NAG-thiazoline was relatively limited compared with that of the GH18 or GH20 enzymes (27). Glu-441 of Endo-D, corresponding to Asp-279 of Endo-M, was found to be hydrogen bonding with O6 of NAG-thiazoline (27). These results suggested that Asp-279 of Endo-M is important for recognition and efficient uptake of the donor substrate.

On the basis of these observations, we propose here a plausible mechanism of Endo-M involving Asn-175, Glu-177, and Asp-279 (Fig. 5C) as follows. (i) Asp-279 recruits the substrate by forming a hydrogen bond to O6 of the donor substrate to properly fit it in the active site and then the Glu-177 protonates the oxygen of the β1,4-glycosidic bond in the substrate to activate the anomic center, whereas the tautomeric Asn-175 ori-2-acetamide to promote the formation of the oxazolinium ion intermediate. (ii) Asn-175 may aid Glu-177 to activate the acceptor/water molecule for the oxazoline processing to transglycosylation/hydrolysis.

Considering practical application using sugar oxazoline, N175Q holds a great advantage, as it enables high yield syntheses with a much smaller amount of enzyme and a much shorter incubation time than N175A. Enhancing the reaction is particularly important as the sugar oxazoline can be gradually hydrolyzed in an aqueous solution during a prolonged incubation. The exceptional catalytic efficiency of N175Q was exemplified by the high yield chemoenzymatic synthesis of two defined glycoforms of CD52 by using either high mannose-type or complex-type sugar oxazoline (Scheme 2). Moreover, it was also found that N175Q enabled efficient synthesis using “natural” N-glycan substrate as a donor (Scheme 3). In this case, a large amount of the enzyme and long incubation time are necessary. Nevertheless, N175Q is still much superior to Y217F for practical use; the maximum yield of the synthetic product by each enzyme was comparable, but the donor consumption was much more reduced in N175Q (30%) than in Y217F (50%) (Fig. 4C). Therefore the productivity of N175Q is estimated to be 1.5-fold higher than that of Y217F. Most importantly, the hydrolysis of the product by N175Q was much slower than that of Y217F (Fig. 4B). In this sense, the N175Q behaves as a typical transglycosidase. The novel transglycosylation activity of N175Q with both activated sugar oxazoline and ground-state natural N-glycans significantly expands the donor substrate repertoire for oligosaccharide and glycoconjugate synthesis.

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