Mutants of Mucor hiemalis Endo-β-N-acetylglucosaminidase Show Enhanced Transglycosylation and Glycosynthase-like Activities*

Received for publication, August 27, 2007, and in revised form, December 4, 2007. Published, JBC Papers in Press, December 20, 2007, DOI 10.1074/jbc.M707137200

Midori Umekawa†, Wei Huang†, Bing Li†, Kiyotaka Fujita†, Hisashi Ashida†, Lai-Xi Wang†, §, and Kenji Yamamoto†, §

From the Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kitashirakawa, Sakyoku, Kyoto 606-8502, Japan, the Institute of Human Virology and Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland 21201, and the Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan

Endo-β-N-acetylglucosaminidase from Mucor hiemalis (Endo-M), a family 85 glycoside hydrolase, acts on the B1,4 linkage of N,N’-diacetyltetritolbiose moiety in the N-linked glycans of glycoproteins and catalyzes not only the hydrolysis reaction but also the transglycosylation reaction that transfers the releasing sugar chain to an acceptor other than water to form a new glycosidic linkage. The transglycosylation activity of Endo-M holds great promise for the chemo-enzymatic synthesis and glyco-engineering of glycoproteins, but the inherent hydrolytic activity for product hydrolysis and low transglycosylation have hampered its broad applications. This paper describes the site-directed mutagenesis on residues in the putative catalytic region of Endo-M to generate mutants with superior transglycosylation activity. Two interesting mutants were discovered. The Y217F mutant was found to possess much enhanced transglycosylation activity and yet much diminished hydrolytic activity in comparison with the wild-type Endo-M. Kinetic analysis revealed that the $K_m$ value of Y217F for an acceptor substrate 4-methylumbelliferyl-β-D-N-acetylglucosaminide was only one-tenth of that of the wild-type, implicating a much higher affinity of Y217F for the acceptor substrate than the wild-type. The other mutant, N175A, acts like a glycosynthase. It was found that mutation at Asn175”knocked out” the hydrolytic activity, but the mutant was able to take the highly active sugar oxazolines (the transition state mimics) as donor substrates for transglycosylation. This is the first glycosynthase derived from endo-β-N-acetylglucosaminidases that proceed via a substrate-assisted mechanism. Our findings provide further insights on the substrate-assisted mechanism of GH85. The usefulness of the novel glycosynthase was exemplified by the efficient synthesis of a human immunodeficiency virus, type 1 (HIV-1) glycopeptide with potent anti-HIV activity.

Endo-β-N-acetylglucosaminidase (EC 3.2.1.96) (ENGase) catalyzes hydrolysis of the B1,4-glycosidic linkage of the N,N’-diacetyltetritolbiose moiety in the core of asparagine-linked glycan of various glycoproteins and glycopeptides. This type of enzyme is widely distributed in animals, plants, fungi, and bacteria. Several bacterial enzymes, such as Endo-H from Streptomyces plicatus (1) and Endo-F, from Flavobacterium meningosepticum (2), were cloned and classified into glycoside hydrolase (GH) family 18 in the CAZy data base (available on the World Wide Web), which may share a common evolutionary origin with GH18 chitinases. The other ENGases are distinct from the enzymes of the GH18 chitinase family and are classified into the GH family 85. We and others have previously reported that several ENGases of the GH85 family showed significant transglycosylation activity (i.e. the ability to transfer the releasing glycan to an acceptor other than water to form a new glycosidic linkage) (3–6). These ENGases include Endo-M from Mucor hiemalis (3), Endo-A from Arthrobacter protophormiae (4), Endo-Ce from Caenorhabditis elegans (5), and Endo-BH from alkaliphilic Bacillus halodurans C-125 (6). Whereas Endo-A, Endo-Ce, and Endo-BH are mainly specific for high mannose type N-glycans, the Endo-M is unique in that it can act on all three types of N-glycans (high mannose type, complex type, and hybrid type) for hydrolysis and transglycosylation. The transglycosylation activity of the ENGases, specifically Endo-M and Endo-A, has found useful applications for the synthesis of novel oligosaccharides and glycoconjugates (7–9). For example, we have applied the Endo-M-catalyzed transglycosylation for the synthesis of complex glycopeptides and for the glycosylation remodeling of glycoproteins (10–14). The chemo-enzymatic method provides a highly convergent synthesis of glycopeptides, since the ENGases are able to attach a large oligosaccharide moiety to a preformed GlcNAc-peptide

* This work was supported in part by the 21st Century Centers of Excellence Program of the Japan Society for the Promotion of Science (to the Graduate School of Biostudies and Institute for Virus Research, Kyoto University), by Core Research for Evolutional and Synthetic Science and Technology of the Japan Science and Technology Agency, and by National Institutes of Health Grants R01 GM 073717 and R01 GM080374 (to L. X. W.). The costs of publication of this article were defrayed in part by the payment of page charges. The authors declare no competing financial interests.

† To whom correspondence may be addressed: Institute of Human Virology and Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 725 W. Lombard St., Baltimore, MD 21201. Tel.: 410-706-4982; Fax: 410-706-5068; E-mail: lwang3@ihv.umaryland.edu.

§ To whom correspondence may be addressed. Tel: 81-75-753-6277; Fax: 81-75-753-9228; E-mail: yamamoto@kais.kyoto-u.ac.jp.

3 The abbreviations used are: ENGase, endo-β-N-acetylglucosaminidase; Endo-M, endo-β-N-acetylglucosaminidase from M. hiemalis; GH, glycoside hydrolase; SGP, the biantennary complex type sialylglycopeptide; ESI-MS, electron spray ionization-mass spectrometry; 4MU-GlcNAc, 4-methylumbelliferyl-β-D-N-acetylglucosaminide; HPLC, high performance liquid chromatography; MS, mass spectrometry; HIV, human immunodeficiency virus.
Enhanced Transglycosylation by Endo-M Mutants

in a single step and in a regio- and stereo-selective manner without the need of protecting groups. Nevertheless, since ENGases are inherently glycohydrolyases, a broader application of the endoglycosidasases for synthetic purposes has been hampered by their relatively low transglycosylation activity and by the issue of product hydrolysis. Although the incorporation of organic solvents in the reaction medium can enhance the transglycosylation yield to some extent (15), the overall efficiency is generally low. In addition, the limitation to the use of only natural N-glycans or N-glycopeptides as the donor substrates further constrains the usefulness of the approach. As an effort to address these problems, we and others have recently explored synthetic sugar oxazolines, a highly active species mimicking the presumed transition state, as donor substrates for the enzymatic transglycosylation (16-22). It was found that this strategy was particularly useful for the synthesis of glycopeptides carrying truncated and/or modified N-glycans, since the highly activated sugar oxazolines could tolerate certain modifications, but the resulting (ground state) glycopeptide product would become resistant to enzymatic hydrolysis due to the slight modification, allowing accumulation of the product (17, 19, 20, 22). However, when glycopeptides and glycoproteins carrying natural, full-size N-glycans are concerned, the rapid hydrolysis of the product by the wild-type endoglycosidasases is difficult to avoid, thus limiting its wide application. An ultimate solution to this problem is perhaps to develop unique ENGase-based glycosynthases that lack hydrolysis activity to the product but are able to take highly activated species as donor substrate (23, 24).

For this purpose, we have performed site-directed mutagenesis of Endo-M as a way to enhance its transglycosylation activity and/or to diminish its hydrolysis activity, based on preliminary structural information and catalytic mechanism of ENGases of the GH85 family. In this paper, we describe two interesting mutants of Endo-M; one shows significantly enhanced transglycosylation activity, and the other shows glycosynthase-like activity when the activated sugar oxazoline is used as the donor substrate.

EXPERIMENTAL PROCEDURES

Materials—The biantennary complex-type sialylglycopeptide (SGP), Lys-Val-Ala-Asn((NeuAc-Gal-GlcNAc-Man)Man-GlcNAc)2-Lys-Thr was prepared from hen egg yolks following the reported procedure (13, 25); Man,GlcNAc2-Aasn was prepared from soy bean flour according to our modified procedure (26); the GlcNAc-pentapeptide (Glu-Asn(GlcNAc)-Ile-Thr-Val) (1) derived from chitinase from S. griseus (45 mg, 18.8 μmol) was prepared from soy bean flour by the previously described procedure (26), was dissolved in the mixture of pyridine (3 ml) and acetic anhydride (3 ml), and the solution was stirred at room temperature for 20 h. The reaction mixture was concentrated under vacuum to dryness, and the residue was subject to column chromatography on silica gel with elutes of CH2Cl2/MeOH (20:1) to afford the fully acetylated Man, derivative as a pale yellow syrup (56 mg) (yield, 70%). ESI-MS: calculated, M = 2983.6; found, 2984.3 [M + H]+, 1492.7 [M + 2H]2+. The acetylated Man, derivative (5) (56 mg, 18.8 μmol) was dissolved in anhydrous 1,2-dichloroethane (4 ml), and then TMS-Br (40 μl, 0.33 mmol), BF3·Et2O (40 μl, 0.33 mmol), and 2,4,6-collidine (47 μl, 0.33 mmol) were added sequentially under an argon atmosphere. The mixture was stirred at room temperature overnight and then diluted with chloroform (20 ml) and washed with saturated sodium bicarbonate solution and brine. The organic layer was dried over anhydrous sodium sulfate and filtered, and the filtrate was concentrated. The residue was purified by column chromatography on silica gel with the eluate (CH2Cl2/MeOH, 20:1) to give a crude product as a yellow solid. The crude product was further purified by gel filtration (Sephadex LH-20, eluted with MeOH) to afford acetylated Man,GlcNAc-oxazoline derivative as a pale yellow foam (40 mg, 74%). 1H NMR of the acetylated Man,GlcNAc-oxazoline (6) (56 mg, 0.33 mmol) was dissolved in anhydrous pyridine (3 ml), and acetic anhydride (3 ml), and the solution was stirred at room temperature for 20 h. The reaction mixture was concentrated under vacuum to dryness, and the residue was subject to column chromatography on silica gel with elutes of CH2Cl2/MeOH (20:1) to afford the fully acetylated Man, derivative as a pale yellow syrup (56 mg, 74%). 1H NMR of the acetylated Man,GlcNAc-oxazoline derivative: (CDCl3, 500 MHz-): δ 5.92 (d, J = 7.0 Hz, 1H, H-1 of oxazoline), 5.51 (s, 1H), 5.45 (s, 1H), 5.39-4.78 (m, 30H), 4.22–3.52 (m, 37H), 2.24–2.03 (m, 93H, 30 Me on acetyl group and 1 Me on oxazoline); ESI-MS: calculated, M = 2923.5; found, 2924.2 [M + H]+, 1462.6 [M + 2H]2+. The acetylated Man,GlcNAc-oxazoline (40 mg, 13.7 μmol) was treated with MeONa in anhydrous MeOH (0.5 mm, 4 ml) for 16 h, and the de-O-acetylation was monitored by ESI-MS. The MeOH was removed by evaporation, and the residue was dissolved in water and subjected to gel filtration (Sephadex G-10, eluted with water containing 0.03% Et3N). The fractions containing the product were combined and lyophilized to give the acetylated Man,GlcNAc-oxazoline (6) as a pale yellow solid (24 mg, quantitative yield). 1H NMR (D2O, 500 MHz-): δ 6.03 (d, J = 7.0 Hz, 1H, H-1 of oxazoline), 5.31 (s, 1H), 5.26 (s, 1H), 5.23 (s, 1H), 4.98 (s, 3H) 4.85 (s, 1H), 4.65 (s, 1H), 4.10 (s, 1H), 4.06–3.33 (m, 58H), 1.84 (s, 3H, Me of oxazoline); ESI-MS: calculated, M = 1661.5; found, 1680.2 [M + H]+, 1662.2 [M + H]+, 1500.1 [M + H]+, 842.7 [M + H + Na]2+, 852.0 [M + H + Na + H2O]2+, 761.6 [M + H + Na + Na2]+.

Expression and Purification of Recombinant Endo-M in Escherichia coli—The cDNA fragment encoding the wild-type Endo-M was amplified by PCR using chromosomal DNA extracted from the recombinant Candida boidinii producing Endo-M (27) with a forward primer, 5’-GGAATTCATATG-CCTTCACTTCAATTGCA-3, and a reverse primer, 5’-CCG CTCCGACGTTAATGCAACAATCTATGCT-3’. Ndel and XhoI sites (underlined) were added to the forward and reverse primers, respectively. The amplified fragment was digested with Ndel and XhoI and subcloned into pET23b+ (vector) (Novagen) to fuse a His6 tag at the C terminus. E.coli BL21(DE3) was transformed with the constructed plasmid (pET23b-Endo-M), and the transformants were cultured in Luria-Bertani medium containing 100 mg/liter ampicillin at 20 °C for 38 h to express Endo-M slowly. The cells were collected and resuspended in BugBuster Protein Extraction Reagent (Novagen) containing 1 mM phenylmethylsulfonyl fluo-
ride, and insoluble materials were removed by centrifugation. Clear supernatant obtained was applied onto Ni2+-charged HiTrap chelating column (GE Healthcare). Unbound proteins were washed with the binding buffer (20 mM sodium phosphate, pH 7.4) containing 0.5 M NaCl and 10 mM imidazole. Endo-M was eluted with the elution buffer (20 mM sodium phosphate, pH 7.4) containing 0.5 M NaCl and 100 mM imidazole. The eluate was desalted and concentrated with Amicon Ultra filtration (10,000, Millipore).

**Site-directed Mutagenesis**—The mutants were generated by PCR using KOD-plus DNA polymerase (Toyobo, Japan) and pET23b-Endo-M as a template according to the procedure for a QuikChange site-directed mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing. The plasmids containing mutated Endo-M genes were introduced into E. coli BL21 (DE3). Expression and purification of mutants were carried out in the same way as for the wild-type enzyme. Homogeneity of each purified enzyme was confirmed by SDS-PAGE/Coomassie Brilliant Blue staining.

**Enzyme Assay**—Assay for the hydrolysis activity of Endo-M was performed with 1 mM dansylated SGP, which was prepared by the method of Gray (28), as a substrate in 50 mM sodium phosphate buffer (pH 6.25, 20 μl) at 30 °C, and the reaction was stopped by boiling for 3 min. The reaction mixtures were analyzed by reverse-phase high performance liquid chromatography (HPLC) using a Cosmosil 5C18-AR II column (4.6 × 150 mm; Nacalai Tesque, Japan) on a HPLC system equipped with an L-2480 FL detector. Elution was carried out with 50% MeCN in 25 mM sodium borate buffer (pH 7.5) at a flow rate of 0.5 ml/min. Fluorescence of dansyl group was detected with an excitation wavelength of 320 nm and an emission wavelength of 540 nm. One unit of the enzyme was defined as the amount of protein yielding 1 μmol of dansylated peptide-GlcNAc/min at 30 °C.

An assay for the transglycosylation activity was carried out according to the method previously described (29). Briefly, the reaction mixture containing 1 mM SGP, 2.5 mM 4MU-GlcNAc, 100 milligrams of chitinase from S. griseus, 10% dimethyl sulfoxide, and 0.7 μg of the wild-type Endo-M or the mutant was incubated at 30 °C in 50 mM sodium phosphate buffer (pH 6.25, 50 μl). Aliquots (5 μl) were added to 150 mM glycine buffer (pH 10.5, 200 μl) to stop the reaction. Fluorescence was measured using a microplate reader (Power Scan HT, Dainippon Sumitomo Pharma, Japan) (excitation, 355 nm; emission, 460 nm). One unit of the enzyme was defined as the amount of enzyme yielding 1 μmol of 4MU/min.

The transglycosylation reaction toward GlcNAc was carried out as follows. 1 mM SGP, 50 mM GlcNAc, and 0.8 μg of the wild-type Endo-M or 1.4 μg of the Y217F mutant was incubated in 25 mM ammonium acetate (pH 6.0, 80 μl) at 30 °C. Reaction mixtures were analyzed by TLC on silica gel 60 plate (Merck) with a solvent system of 1-propanol/acetic acid/water (3:3:2, v/v/v). Sugars were visualized by heating after spraying on the plates with orcinol-H2SO4 reagent. The amounts of the product and the substrate were quantified by the image analyzer (Multi Gauge version 2.2).

**Transglycosylation to GlcNAc-pentapeptide**

**Transglycosylation to GlcNAc-pentapeptide (1)** Using the Complex Type Natural Glycopeptide SGP as the Donor Substrate—A solution of SGP (1.72 mg, 0.6 μmol) and the GlcNAc-pentapeptide derived from erythropoietin (1) (0.16 mg, 0.2 μmol) (molar ratio of donor/acceptor, 3:1) in 50 mM sodium phosphate buffer (pH 6.6, 50 μl) was incubated with 3.5 μg of the wild-type Endo-M or the mutant Y217F at 30 °C. Aliquots (2 μl each) were taken at intervals and heated at 100 °C for 3 min to stop the reaction. The aliquots were then diluted and were subject to reverse-phase HPLC analysis. The analytical HPLC was performed on a Waters Nova-Pak C18 column (3.9 × 150 mm) at 40 °C. The column was eluted with a linear gradient of 0–30% MeCN containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min over 18 min. Peptides and glycopeptides were detected at UV 214 nm. The yield of transglycosylation was calculated by integration of the peaks and normalized with the absorbance: transglycosylation yield (%) = (product/(product + remaining acceptor)) × 100. For preparative purpose, the product was purified by preparative HPLC with a Waters C18 column (19 × 300 mm; Symmetry 300). The column was eluted with a suitable gradient of MeCN containing 0.1% trifluoroacetic acid at a flow rate of 12 ml/min. Glycopeptide product 2: analytical HPLC: tR = 13.4 min (linear gradient of 0–30% MeCN containing 0.1% trifluoroacetic acid in 18 min, flow rate 1 ml/min); ESI-MS: calculated, M = 2778.1; found, 1390.7 [M + 2H]2+, 927.8 [M + 3H]3+.

**Transglycosylation to GlcNAc-Pentapeptide (1) Using the Natural Oligosaccharide Man6GlcNAcAsn as the Donor Substrate**—A solution of Man6GlcNAc2-Asn (1.5 mg, 0.75 μmol) and the GlcNAc-pentapeptide derived from erythropoietin (1) (0.19 mg, 0.25 μmol) (donor/acceptor, 3:1) in 50 mM sodium phosphate buffer (pH 6.6, 50 μl) was incubated with 2.0 μg of the wild-type Endo-M or the mutant Y217F at 30 °C. Aliquots (2 μl each) were taken at intervals and heated at 100 °C for 3 min to stop the reaction. The aliquots were then diluted and were subject to reverse-phase HPLC analysis, as described above. Glycopeptide product 3: analytical HPLC, tR = 13.8 min (linear gradient of 0–30% MeCN containing 0.1% trifluoroacetic acid in 18 min, flow rate 1 ml/min); ESI-MS: calculated, M = 2419.1; found, 1220.5 [M + 2H]2+, 1139.4 [M−Man + 2H]2+. Further structural characterization of product was performed by Endo-H hydrolysis. The product was treated with Endo-H to give a GlcNAc-containing peptide and an oligosaccharide. The GlcNAc-containing peptide was identical to the authentic GlcNAc-pentapeptide (1), and the oligosaccharide was identical to the standard Man6GlcNAc as measured by MS, HPLC, and Dionex high performance anion exchange chromatography with pulsed electrochemical detection analysis. In addition, digestion of Man6GlcNAc2-pentapeptide with Pronase gave Man5GlcNAc2-Asn.

**Transglycosylation to GlcNAc-pentapeptide (1) Using Synthetic Sugar Oxazolines as the Donor Substrate**—A solution of Man6GlcNAc-oxazoline (6) (1.25 mg, 0.75 μmol) and GlcNAc-pentapeptide (1) (0.29 mg, 0.38 μmol) in 50 mM sodium phosphate buffer (pH 6.6, 30 μl) was incubated with the wild-type Endo-M (3.5 μg), the mutant Y217F (3.5 μg), or the mutant N175A (20 μg) at 30 °C. Aliquots were taken at intervals and were analyzed by HPLC as described above. For the reaction with the truncated form of sugar oxazoline (7), a solution of Man5GlcNAc-oxazoline (7) (0.66 mg, 0.96 μmol) and GlcNAc-
Enhanced Transglycosylation by Endo-M Mutants

pentapeptide (1) (0.33 mg, 0.43 μmol) in 50 mM sodium phosphate buffer (pH 6.6, 30 μl) was incubated with the wild-type Endo-M (3.5 μg), the mutant Y217F (3.5 μg), or the mutant N175A (10 μg) at 30 °C. Aliquots were taken at intervals and were analyzed by HPLC as described above. Man3GlcNAc2-pentapeptide (8): analytical HPLC: t₂₅ = 14.1 min (linear gradient of 0–30% MeCN containing 0.1% trifluoroacetic acid in 18 min, flow rate at 1 ml/min); ESI-MS: calculated, M = 1466.2; found, 1467.0 [M + H]⁺, 734.3 [M + 2H]²⁺.

Synthesis of HIV-1 gp41 Glycopeptide Man₅GlcNAc₂-C34 (10) Using Mutant N175A—To a solution of GlcNAc-C34 (9) (0.55 mg, 0.11 μmol) in 50 mM sodium phosphate buffer (pH 6.6, 35 μl) containing 15% dimethyl sulfoxide was added Man₅GlcNAc-oxazoline (6) (1.0 mg, 0.6 μmol). The mixture was incubated with N175A (5 μg) at 30 °C. After 12 h, the reaction was quenched by adding with 1% trifluoroacetic acid (10 μl). The mixture was lyophilized, and the residue was subject to preparative HPLC to give the product Man₅GlcNAc₂-C34 (10) (0.49 mg, 72%): analytical HPLC: t₂₅ = 17.2 min (linear gradient of 0–90% MeCN containing 0.1% trifluoroacetic acid in 25 min, flow rate 1 ml/min); ESI-MS: calculated M = 6155.5; found, 1540.5 [M + 4H]³⁺, 1499.6 [M-Man + 4H]³⁺, 1232.3 [M + 5H]⁴⁺, 1200.0 [M-Man + 5H]⁴⁺.

RESULTS

Site-directed Mutagenesis of the Residues in the Putative Catalytic Region of Endo-M—Although the crystal structures of Endo-H, Endo-F₁, and Endo-F₃ of the GH18 ENGases, which do not possess transglycosylation activity, have been solved (30–32), no three-dimensional structures are available for the endoglycosidases of the GH family 85, that show transglycosylation activity. Nevertheless, based on its sequence similarity to the GH85 family members, we have previously performed mutational studies on Endo-A to identify critical residues for the enzymatic activity. For example, the highly conserved Trp216 of Endo-A was found to be essential for its transglycosylation activity but not for its hydrolysis activity (33). Recently, we have identified Glu¹⁷⁷ of Endo-A as the putative proton donor by a chemical rescue experiment (34). The residue is conserved in all of the other members of GH85 and corresponds with Glu¹⁷⁷ in Endo-M, as shown by the comparison of the amino acid sequences of ENGases belonging to GH85 (Fig. 1). Based on this analysis with Endo-A, we assume that the corresponding residues in Endo-M and the surrounding region might also be essential for the enzymatic activity of Endo-M. In order to enhance the transglycosylation activity of Endo-M, we attempted to carry out site-directed mutagenesis of various residues in this putative catalytic region that were conserved for the known ENGases of GH family 85 with transglycosylation activity. More recently, a preliminary x-ray crystallographic study on Endo-A was performed by Dr. van Roey and co-workers. According to the preliminary structure, Endo-A represents a (β/α)₉ TIM-barrel structure (clan GH-K) and shows overall structural similarity with the members of GH families 18 and 20. The preliminary structure also suggests that the residues Asn¹⁷¹, Tyr²⁰⁵, Trp²¹⁶, Asn²¹⁸, Asn²⁴², Phe²⁴³, and Trp²⁴⁴ may locate around the catalytic proton donor Glu¹⁷⁷ and interact with substrate within a putative catalytic cleft of Endo-A. Based on this information, we have carried out site-directed mutagenesis of the corresponding residues in Endo-M and assayed the hydrolysis and transglycosylation activities of these mutants (Table 1). For hydrolysis activity, dansylated SGP was used as the substrate, and the hydrolytic product was quantified by HPLC separation and fluorescent detection. For the analysis of the transglycosylation activity, a sensitive chitinase-coupled assay was applied, which was recently developed by us (29). As expected, the Glu to Ala mutation at residue 177 completely abolished the hydrolysis activity of Endo-M, suggesting that Glu¹⁷⁷ functions as the proton donor in Endo-M. Interestingly, the mutant N175A showed only marginal hydrolysis activity, indicating that Asn¹⁷¹ plays an important role in the catalytic mechanism. All of the other mutants showed relatively reduced hydrolysis activity (19–72% of the activity of the wild-type). For the transglycosylation activity using natural SGP as the donor substrate, only the mutant Y217F exhibited enhanced transglycosylation activity over the wild-type (Table 1). Replacement of the Tyr²¹⁶ to other amino acids other than Phe, however, resulted in abolishment of both hydrolysis and transglycosylation activities (data not shown). W228F, Y217F/W228F, and N230S showed reduced transglycosylation activity, and N249S, Y250N, and W251N almost abolished the transglycosylation activity. Consequently, Y217F was identified as the mutant enzyme showing both enhanced transglycosylation activity and diminished hydrolysis activity.

Mutant Y217F Demonstrates Enhanced Transglycosylation Activity—To further assess the transglycosylation activity of the Y217F mutant, we monitored the time course of transglycosylation reaction, using SGP as the donor substrate and...
TABLE 1
Comparisons of the specific activities of hydrolysis and transglycosylation of the wild-type Endo-M and the mutants

| Mutant        | Specific hydrolysis activitya | Percentage of specific hydrolysis activity | Specific transglycosylation activitya | Percentage of specific transglycosylation activity |
|---------------|-------------------------------|------------------------------------------|--------------------------------------|-----------------------------------------------|
|               | μmol min⁻¹ mg⁻¹ | %                             | μmol min⁻¹ mg⁻¹ | %                          |
| Wild type     | 1.6                          | 100                          | 0.058                               | 100                                          |
| N175A         | 0.30 × 10⁻³                  | 0.024                        | ND                                  | ND                                           |
| E177A         | ND                           | ND                          | ND                                  | ND                                           |
| Y217F         | 1.2                          | 72                           | 0.085                               | 147                                          |
| W228F         | 0.46                         | 29                           | 0.026                               | 45                                           |
| Y217F/W228F   | 0.62                         | 38                           | 0.023                               | 40                                           |
| N230S         | 0.70                         | 44                           | 0.023                               | 39                                           |
| N249S         | 0.32                         | 20                           | 0.0017                              | 3.0                                          |
| Y250N         | 0.30                         | 19                           | 0.0027                              | 4.7                                          |
| W251N         | 0.43                         | 27                           | 0.0018                              | 3.1                                          |

a The hydrolysis activity was determined using dansylated SGP as the substrate; the transglycosylation activity was measured using a chitinase-coupled assay, in which SGP was used as the donor substrate and 4MU-GlcNAc was the acceptor.

As to the hydrolysis of the donor substrate, the differences in kinetic parameters for the wild type and the Y217F mutant were not significant; the \( K_m \) for the Y217F mutant (0.30 mM) was more than 10 times lower than that of the wild-type (4.1 mM). As to the acceptor substrate 4MU-GlcNAc, the \( k_{cat}/K_m \) values for the wild type and the mutant were 7.5 × 10⁻³ and 6.0 × 10⁻² mm⁻¹ s⁻¹, respectively.

The remaining donor substrate SGP was shown as follows. Open squares, wild-type Endo-M; closed triangles, mutant Y217F. Yield, the amount of transglycosylation product per initial acceptor substrate.

4MU-GlcNAc as the acceptor in the presence of the coupled enzyme chitinase (29). As a result, the transglycosylation of the Y217F mutant increased linearly at least up to 120 min, whereas that of the wild type reached a plateau after 60 min (Fig. 2A). At 120 min of reaction, the accumulated transglycosylation product by the Y217F mutant was about 5 times that of the wild-type Endo-M, which was quantified by the chitinase-catalyzed release of 4MU from the transglycosylation product (Fig. 2A).

For comparison, the kinetic parameters of the wild type and the Y217F mutant for the acceptor 4MU-GlcNAc were then estimated by using the chitinase-coupled method. The values of the \( k_{cat} \) for the two enzymes were close: 0.031 and 0.018 s⁻¹ for the wild type and the Y217F mutant, respectively. In contrast to their similar \( k_{cat} \) values, the \( K_m \) values for the two enzymes were significantly different; the \( K_m \) for the Y217F mutant (0.30 mM) was more than 10 times lower than that of the wild-type (4.1 mM). As to the acceptor substrate 4MU-GlcNAc, the \( k_{cat}/K_m \) values for the wild type and the mutant were 7.5 × 10⁻³ and 6.0 × 10⁻² mm⁻¹ s⁻¹, respectively.

These results suggest that the enhancement of the transglycosylation activity of the Y217F mutant might be caused by its decreased \( K_m \) for the acceptor substrate in the transglycosylation reaction.

Next, we analyzed the ratio of transglycosylation/hydrolysis by incubating SGP with equal catalytic units of the enzyme in the presence of 50 mM GlcNAc and carrying out TLC. Sugars were visualized with orcinol-H₂SO₄ (Fig. 2B), and we quantified the transglycosylation product/hydrolysis product (\( T/H \)) ratio using an image analyzer. According to the results, the amount of the transglycosylation product of the wild type was initially greater than that of the hydrolysis product (\( T/H \) ratio at 30 min, 1.2), but after depletion of SGP (120 min or later), it gradually decreased due to rehydrolysis, and the \( T/H \) ratio decreased (1.0 at 120 min and 0.7 at 240 min). On the other hand, the \( T/H \) ratio of the Y217F mutant was 1.7 at 30 min, and the ratio remained...
high, compared with that of the wild type, until depletion of SGP (1.7 at 120 min and 1.3 at 240 min).

The transglycosylation efficiencies of the mutant Y217F and the wild-type Endo-M for \( \text{N-glycopeptide synthesis} \) were compared, using a GlcNAc-containing pentapeptide (Glu-Asn(GlcNAc)-Ile-Thr-Val) derived from the erythropoietin (amino acid sequence 37–41) as the acceptor and the complex type (SGP) and high mannose type oligosaccharide (Man9GlcNAc2-Asn) as the donor substrates, respectively (Scheme 1). The ratio of the donor substrate and the acceptor was set at 3:1, and the enzymatic reaction was monitored by HPLC analysis. When SGP was used as the donor substrate under the given reaction conditions, it was observed that the transglycosylation product from the wild-type Endo-M-catalyzed reaction reached the maximum (about 25%) at 80 min (Fig. 2C), where half of the donor substrate was consumed. Thereafter, the transglycosylation product started to decrease because of its hydrolysis, and at 400 min, the transglycosylation product was almost completely hydrolyzed. In the case of the mutant Y217F, the rate of the total consumption of the donor substrate SGP was much slower than that of the wild type, and the yield of the transglycosylation product continuously increased over 400 min. It approached the maximum yield of ~50% at 400 min (Fig. 2C). When slightly modified sugar oxazolines of the N-glycans were used, since the resulting glycopeptide product became resistant to hydrolysis due to the slight modification, whereas the modified sugar oxazolines could still serve as excellent donor substrates because of their highly activated nature and their mimicking of the transition state. However, when applied to glycopeptides with full-size, natural N-glycans, the enzymatic hydrolysis of the resulting glycopeptide products, which are excellent substrates for respective wild-type ENGases, would become an unavoidable problem. To examine the transglycosylation potential of the Endo-M mutants using sugar oxazolines corresponding to native N-glycans, we first synthesized sugar oxazoline (6) corresponding to the natural high mannose type N-glycan Man\(_{9}\)GlcNAc\(_{2}\)-Asn (Scheme 2). Man\(_{9}\)GlcNAc\(_{2}\)-Asn from soybean flour was hydrolyzed by Endo-A to give the oligosaccharide Man\(_{9}\)GlcNAc (4), which was easily isolated by Sephadex G-15 gel filtration. Acetylation of 4 with acetic anhydride/pyridine gave the fully acetylated Man\(_{9}\)GlcNAc (5). Compound 5 was then treated with BF\(_{3}\)/Et\(_{2}\)O and trimethylsilyl bromide in the presence of collidine to provide the corresponding \( O\)-acylated sugar oxazoline derivative, which was subject to \( de-O\)-acylation with MeONa/MeOH to afford the Man\(_{9}\)GlcNAc-oxazoline (6) (Scheme 2). The synthetic
Man$_9$GlcNAc-oxazoline was used as the donor substrate to examine the transglycosylation with the wild-type Endo-M and its mutants, where GlcNAc-pentapeptide (1) was used as the acceptor (donor/acceptor, 2:1). It was found that the mutant Y217F exhibited much higher transglycosylation activity toward the Man$_9$GlcNAc-oxazoline than the wild-type Endo-M. Under the reaction conditions, the yield of the transglycosylation product reached 50% for the mutant Y217F, whereas the wild-type Endo-M gave only 8% of the transglycosylation product at the maximum. As expected, the product was gradually hydrolyzed in both cases, given a prolonged incubation time (Fig. 3A). When a smaller, tetrasaccharide Man$_3$GlcNAc-oxazoline was used as the donor substrate, which is a truncated form of the corresponding core N-glycan, the Y217F-catalyzed transglycosylation also occurred smoothly and gave a much higher yield of the product (8) (70%) than that of the reaction using Man$_9$GlcNAc-oxazoline as the donor, due to the decreased hydrolysis of the truncated form of N-glycan by the enzyme (Scheme 3 and Fig. 3B).

Mutant N175A Exhibits Glycosynthase-like Activity—Although the Y217F mutant possessed effective transglycosylation activity with sugar oxazolines, the transglycosylation products were still subject to enzymatic hydrolysis during transglycosylation. This is particularly true when the N-glycans attached are excellent substrates for the enzyme. To search for glycosynthase-like mutants that lack hydrolysis activity toward the product, we have examined the mutants that are inactive for hydrolysis of N-glycans (Table 1) for transglycosylation activity. Interestingly, it was found that the N175A mutant could take N-glycan oxazolines as the donor substrates for transglycosylation to form the corresponding glycopeptides (Fig. 3, A and B). As expected, the transglycosylation product, once formed, is resistant to enzymatic hydrolysis. Thus, in combination with the highly activated sugar oxazoline as the donor substrate, the mutant N175A acted as a glycosynthase for glycosidic bond formation, allowing the accumulation of the transglycosylation product. It was also found
Efficient Synthesis of HIV-1 C34 Glycopeptide Using Mutant N175A—The glycosynthase-like activity of N175A opens a way for synthesizing large, homogeneous N-glycopeptides carrying native N-glycans. We have applied the N175A mutant for the synthesis of Man$_9$GlcNAc$_2$-C34, a 34-mer glycopeptide derived from HIV-1 gp41 that carries a native high mannose N-glycan (Scheme 4). Man$_9$GlcNAc$_2$-C34 was shown to be a potent HIV entry inhibitor (IC$_{50}$ = 12 nM) (35). In contrast to the parent polypeptide C34, Man$_9$GlcNAc$_2$-C34 has much higher water solubility than C34 (35), thus having a potential to be developed as an anti-HIV drug. The glycopeptide was previously synthesized in about 10% yield by Endo-A-catalyzed transglycosylation using 5-fold excess of Man$_9$GlcNAc$_2$-Asn as the donor substrate (35). Here we found that when Man$_9$GlcNAc-oxazoline was used as the donor substrate, the N175A-catalyzed transglycosylation to GlcNAc-C34 (9) proceeded smoothly to give a much higher yield of the product Man$_9$GlcNAc$_2$-C34 (10) (72%), using only a 2-fold excess of the donor substrate. It was also found that N175A could tolerate the use of dimethyl sulfoxide as a co-solvent that helped to improve the solubility of the acceptor GlcNAc-C34 in the reaction buffer.

DISCUSSION

In this study, we generated and focused on two Endo-M mutants, Y217F and N175A, which exhibited different behaviors of transglycosylation. The Y217F mutant catalyzed the transglycosylation reaction more efficiently than the wild type. Kinetic analysis revealed that the Y217F mutant showed much higher affinity for the GlcNAc acceptor than the wild type, as indicated by its much lower $K_m$ value for 4MU-GlcNAc. These results suggest that the removal of the hydroxyl group at Tyr$_{217}$ (i.e., the change of the Tyr residue to a Phe residue) might reduce the steric hindrance and hydrophilicity, thus favoring the interaction and accommodation of the GlcNAc moiety in the acceptor at the catalytic site. Another explanation is that the hydroxyl group on the Tyr$_{217}$ residue might promote hydrolysis via coordinating with the incoming water at the catalytic site. Thus, removing the hydroxyl group on the Tyr residue would result in a diminished hydrolysis activity of the enzyme. Indeed,
the demonstrated reduction of hydrolysis activity of the Y217F mutant over the wild type (Table 1 and Fig. 2) supports this notion. We have also found that the Y205F mutant of Endo-A (corresponding to Y217F of Endo-M) demonstrated enhanced transglycosylation activity over the wild-type Endo-A (data not shown). On the other hand, substitutions of the ‘Tyr’ in Endo-M with amino acid residues other than a Phe residue resulted in loss of both hydrolysis and transglycosylation activities. These results suggest that the phenyl group at this position is important both for acceptor recognition and for catalytic activities. It was demonstrated that the Y217F mutant was significantly more efficient than the wild-type Endo-M for transglycosylating both complex type and high mannose type oligosaccharides to form new, homogeneous N-glycopeptides (Fig. 2).

On the other hand, our mutational analysis also indicates that the Asn175 and Glu177 in Endo-M are essential residues for the catalytic activity of the enzyme. The Glu177 residue most likely serves as the catalytic proton donor residue, since the corresponding residue in Endo-A (Glu173) was characterized as the proton donor for catalysis (33, 34). In our experiments, no transglycosylation was observed in E177A mutant even when the sugar oxazoline was used as donor (data not shown), consistently suggesting that the Glu177 residue is catalytically essential to activate the acceptor or water to bind the oxazolinium ion intermediate. For the GH18 chitinases and GH20 β-N-acetylhexosaminidases, a substrate-assisted catalytic mechanism was proposed, in which the 2-acetamido substituent acts as a nucleophile to form an oxazolinium ion intermediate (36–38). In this substrate-assisted mechanism of the GH families 18 and 20, a conserved acidic residue (usually Asp) located 1 or 2 amino acid residues upstream from the proton donor residue was identified, which assists the appropriate orientation of the 2-acetamido group for catalysis and may also stabilize the transition state flanking the oxazolinium ion intermediate (36, 37). The GH85 enzymes might also follow a similar substrate-assisted mechanism, since some of the GH85 enzymes, such as Endo-A and Endo-M, were shown to be able to take synthetic sugar oxazolines as the donor substrates for transglycosylation (16–22). However, the catalytic mechanism of GH85 enzymes could be different in a narrow sense from those of GH18 and GH20. In GH85 enzymes, a highly conserved Asn residue (the Asn175 in Endo-M) may play the same role as the corresponding Asp residue in GH18 chitinase and GH20 β-N-acetylhexosaminidase. Thus, upon protonation on the anomeric oxygen, the Asn175 residue in Endo-M may assist the appropriate orientation of the 2-acetamido group, promoting the nucleophilic attack of 2-acetamido group at the anomeric position to form the oxazolinium ion intermediate that will proceed further to hydrolysis and transglycosylation. Our experiment has demonstrated that mutation of the Asn175 residue to other amino acid residues, such as Ala and Asp, resulted in the abolishment of hydrolytic activity. Interestingly, the N175A mutant is able to recognize the highly active sugar oxazoline as the donor substrate for transglycosylation. Thus, the N175A acts as a novel glycosynthase (i.e. glycosidase) mutant that lacks hydrolytic activity but promotes glycosidic bond formation when an active glycosyl donor such as glycosyl fluoride with an opposite anomeric configuration is used as a substrate (23, 24).

It should be pointed out that a successful generation of glycosynthases from glycosidases depends on several factors, such as an understanding of the enzymatic mechanism, the identification of the catalytic nucleophile, and the design and synthesis of suitable activated glycosyl donors (23, 24). In the case of GH85 ENGases that proceed via the substrate-assisted mechanism, the nucleophile is the 2-acetamido group in the substrate. Therefore, the conventional approach to generate a glycosynthase by mutating the nucleophilic residue to a nonnucleophile cannot be directly applied. Our identification of the Asn residue at position 175 as a critical residue for promoting oxazolinium ion intermediate formation points to a novel approach to creating a glycosynthase from glycosidases that proceed via a substrate-assisted mechanism. The mutant N175A is the first glycosynthase generated from this class of glycoside hydrolases.

The identification of the glycosynthase-like mutant N175A, together with the finding that this novel mutant could use the highly active sugar oxazoline as its substrate for transglycosylation but lacks the product hydrolysis activity, has significantly expanded our repertoire for chemo-enzymatic glycopeptide synthesis and glycoprotein engineering. As exemplified by the efficient synthesis of the HIV-1 gp41 glycoprotein domain C34, the combined use of the novel glycosynthase and synthetic sugar oxazoline allows a high yield synthesis of glycopeptides carrying full-size, natural N-glycan. The novel mutant enzyme promotes the synthesis without hydrolysis of the resulting glycopeptide product. The use of synthetic sugar oxazolines as the donor substrates also significantly expands the substrate availability and scope for the enzymatic transglycosylation, which had hitherto limited to the use of naturally isolated N-glycans and glycopeptides as the donor substrates. In the case of the high mannose type N-glycan, the synthesis of the Man₇GlcNAc₂-oxazoline from Man₇GlcNAc₂Asn took four steps with a satisfactory overall yield (42% in four steps) (Scheme 2). In a practical glycoprotein or glycopeptide synthesis, one of the two substrates (either the sugar oxazoline or the GlcNAc-protein) is usually used in an excess molar amount in order to make full use of the other more precious substrate, and the yield of the N175A-catalyzed transglycosylation is in the range of 50–80% (Fig. 3A). In contrast, the wild type enzyme Endo-M usually gives much lower transglycosylation yield even if the highly active sugar oxazoline is used as the donor substrate (Figs. 2 and 3), and the transglycosylation product can be quickly hydrolyzed by the enzyme if the reaction is not controlled properly. The next step is to synthesize and examine other types of N-glycan oxazolines, such as the complex type and hybrid type N-glycans, to expand the scope of the chemo-enzymatic method. It is expected that sugar oxazolines corresponding to the complex type N-glycans will be good donor substrates of N175A for transglycosylation, since the parent enzyme Endo-M has an intrinsic ability to transfer the complex-type glycans.

Based on these findings with mutants Y217F and N175A, we propose a plausible mechanism of Endo-M catalysis (Fig. 4). Upon protonation of the glycosidic oxygen by the Glu177, the Asn175 assists an essential orientation of the 2-acetoamido
group in the substrate to promote its nucleophilic attack at the activated anomic center, which will lead to the breakdown of the glycosidic bond and the concurrent formation of the oxazolinium ion intermediate. The resulting oxazolinium ion intermediate is then subjected to an attack by water (for hydrolysis) or an acceptor (for transglycosylation). The role of the Tyr\(^{217}\) is likely to accommodate the coming water and the acceptor. Our experiments have shown that changing the Tyr\(^{217}\) to a Phe residue resulted in a decrease of hydrolytic activity and an increase of transglycosylation activity. The enhanced transglycosylation activity could come from the increased affinity of the mutant enzyme for the acceptor substrate, as indicated by the much smaller \(K_m\) value of the Y217F mutant than that of the wild type for the acceptor 4MU-GlcNAc. Our findings provide insights on the substrate-assisted mechanism of GH85 enzymes, but the understanding of the detailed mechanism of Endo-M catalysis will rely on an x-ray crystallographic study of the enzyme, which is currently under way. The glycosynthase described in this report should be particularly useful for chemo-enzymatic synthesis of large, homogeneous glycopeptides and for glycoengineering of biologically important glycoproteins.

Acknowledgments—We thank Dr. Patrick van Roey and Dr. Kaoru Takegawa for useful suggestions and advice.

REFERENCES

1. Robbins, P. W., Trimble, R. B., Wirth, D. F., Hering, C., Maley, F., Maley, G. F., Das, R., Gibson, B. W., Royal, N., and Biemann, K. (1984) J. Biol. Chem. 259, 7577–7583
2. Tarentino, A. L., Quinones, G., Schrader, W. P., Changchien, L. M., and Plummer, T. H., Jr. (1992) J. Biol. Chem. 267, 3868–3872
3. Yamamoto, K., Kadowaki, S., Watanabe, J., and Kumagai, H. (1994) Biochem. Biophys. Res. Commun. 203, 244–252
4. Takegawa, K., Yamabe, K., Fujita, K., Tabuchi, M., Mita, M., Izu, H., Watanabe, A., Asada, Y., Sano, M., Kondo, A., Kato, I., and Iwahara, S. (1997) Arch. Biochem. Biophys. 338, 22–28
5. Kato, T., Fujita, K., Takeuchi, M., Kobayashi, K., Natsu, S., Ikura, K,
Kumagai, H., and Yamamoto, K. (2002) Glycobiology 12, 581–587
6. Fujita, K., Takami, H., Yamamoto, K., and Takegawa, K. (2004) Biosci. Biotechnol. Biochem. 68, 1059–1066
7. Yamamoto, K., and Takegawa, K. (1997) Trends Glycosci. Glycotechnol. 9, 339–354
8. Wang, L. X., Singh, S., and Ni, J. (2004) in References, pp. 73–92, American Chemical Society, Washington, DC
9. Yamamoto, K. (2006) in Endoglycosidases: Biochemistry, Biotechnology, Application (Endo, M., Hase, S., Yamamoto, K., and Takagaki, K., ed.) pp. 129–140, Kodansha, Tokyo
10. Haneda, K., Inazu, T., Yamamoto, K., Kumagai, H., Nakahara, Y., and Kobata, A. (1996) Carbohydr. Res. 292, 61–70
11. Yamamoto, K., Fujimori, K., Haneda, K., Mizuno, M., Inazu, T., and Kumagai, H. (1998) Carbohydr. Res. 305, 415–422
12. Mizuno, M., Haneda, K., Iuchi, R., Muramato, I., Kawakami, T., Aimoto, S., Yamamoto, K., and Inazu, T. (1999) J. Am. Chem. Soc. 121, 284–290
13. Li, H., Singh, S., Zeng, Y., Song, H., and Wang, L. X. (2005) Bioorg. Med. Chem. Lett. 15, 895–898
14. Fujita, K., and Yamamoto, K. (2006) Biochim. Biophys. Acta 1760, 1631–1635
15. Akaile, E., Tsutsumida, M., Osumi, K., Fujita, M., Yamanoi, T., Yamamoto, K., and Fujita, K. (2004) Carbohydr. Res. 339, 719–722
16. Fujita, M., Shoda, S., Haneda, K., Inazu, T., Takegawa, K., and Yamamoto, K. (2001) Biochim. Biophys. Acta 1528, 9–14
17. Li, B., Zeng, Y., Hauser, S., Song, H., and Wang, L. X. (2005) J. Am. Chem. Soc. 127, 9692–9693
18. Li, H., Li, B., Song, H., Breydo, L., Baskakov, I. V., and Wang, L. X. (2005) J. Org. Chem. 70, 9990–9996
19. Zeng, Y., Wang, J., Li, B., Hauser, S., Li, H., and Wang, L. X. (2006) Chem. Eur. J. 12, 3355–3364
20. Li, B., Song, H., Hauser, S., and Wang, L. X. (2006) Org. Lett. 8, 3081–3084
21. Rising, T. W., Claridge, T. D., Moir, J. W., and Fairbanks, A. I. (2006) ChemBioChem 7, 1177–1180
22. Rising, T. W., Claridge, T. D., Davies, N., Gamblin, D. P., Moir, J. W., and Fairbanks, A. I. (2006) Carbohydr. Res. 341, 1574–1596
23. Perugini, G., Trincone, A., Rossi, M., and Moracci, M. (2004) Trends Biotechnol. 22, 31–37
24. Hancock, S. M., Vaughan, M. D., and Withers, S. G. (2006) Curr. Opin. Chem. Biol. 10, 509–519
25. Seko, A., Koketsu, M., Nishizono, M., Enoki, Y., Ibrahim, H. R., Juneja, L. R., Kim, M., and Yamamoto, T. (1997) Biochim. Biophys. Acta 1335, 23–32
26. Wang, L. X., Ni, J., Singh, S., and Li, H. (2004) Chem. Biol. 11, 127–134
27. Fujita, K., Kobayashi, K., Iwamatsu, A., Takeuchi, M., Kumagai, H., and Yamamoto, K. (2004) Arch. Biochem. Biophys. 432, 41–49
28. Gray, W. R. (1967) Methods Enzymol. 11, 139–152
29. Hauser, S., Song, H., Li, H., and Wang, L. X. (2005) Biochem. Biophys. Res. Commun. 328, 580–585
30. Rao, V., Guan, C., and Van Roey, P. (1995) Structure 3, 449–457
31. Van Roey, P., Rao, V., Plummer, T. H., Jr., and Tarentino, A. L. (1994) Biochemistry 33, 13989–13996
32. Wadding, C. A., Plummer, T. H., Jr., Tarentino, A. L., and Van Roey, P. (2000) Biochemistry 39, 7878–7885
33. Fujita, K., and Takegawa, K. (2001) Biochim. Biophys. Res. Commun. 283, 680–686
34. Fujita, K., Sato, R., Toma, K., Kitahara, K., Suganuma, T., Yamamoto, K., and Takegawa, K. (2007) J. Biochem. (Tokyo) 142, 301–306
35. Wang, L. X., Song, H., Liu, S., Lu, H., Jiang, S., Ni, J., and Li, H. (2005) ChemBioChem 6, 1068–1074
36. Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K. H., Isogai, A., Henriques, B., and Dijkstra, B. W. (1995) Biochemistry 34, 15619–15623
37. Williams, S. J., Mark, B. L., Vocadlo, D. J., James, M. N., and Withers, S. G. (2002) J. Biol. Chem. 277, 40055–40065
38. Vaaje-Kolstad, G., Houston, D. R., Rao, F. V., Peter, M. G., Synstad, B., van Aalten, D. M., and Eijsink, V. G. (2004) Biochim. Biophys. Acta 1696, 103–111

Enhanced Transglycosylation by Endo-M Mutants