Reducing end xylose-releasing exooligoxylanase (Rex, EC 3.2.1.156) is an inverting GH that hydrolyzes xylooligosaccharides ($\geq X_3$) to release $X_1$ at their reducing end. The wild-type enzyme exhibited the Hehre resynthesis hydrolysis mechanism, in which $\alpha$-$X_3F$ was hydrolyzed to $X_2$ and $HF$ in the presence of $X_3$ as an acceptor molecule. However, the transglycosidation product ($X_3$) was not detectable in the reaction. To convert reducing end xylose-releasing exooligoxylanase to glycosynthase, derivatives with mutations in the catalytic base (Asp-263) were constructed by saturation random mutagenesis. Nine amino acid residue mutants (Asp-263 to Gly, Ala, Val, Thr, Leu, Asn, Cys, Pro, or Ser) were found to possess glycosynthase activity forming $X_3$ from $\alpha$-$X_3F$ and $X_1$. Among them, D263C showed the highest level of $X_3$ production, and D263N exhibited the fastest consumption of $\alpha$-$X_3F$. The D263C mutant showed 10-fold lower hydrolytic activity than D263N, resulting in the highest yield of $X_3$. $X_3$ was formed from the early stage of the reaction of the D263C mutant, indicating that a portion of the $X_3$ formed by condensation was hydrolyzed before its release from the enzyme. To acquire glycosynthase activity from inverting enzymes, it is important to minimize the decrease in F$^{-}$-releasing activity while maximizing the decrease in the hydrolytic activity. The present study expands the possibility of conversion of glycosynthases from inverting enzymes.

Glycoside hydrolases (GH) are generally categorized into two types, retaining and inverting enzymes, based on changes in the anomeric configurations during the reactions (1–5). The reaction mechanisms of typical retaining and inverting enzymes are illustrated in Fig. 1. Both types of enzyme have two acidic catalytic residues acting as a general acid (a proton donor) and a general base (a nucleophile). In the retaining GH reaction, first, in concert, the general acid donates a proton to the glycosyl oxygen atom, and the nucleophile attacks the anomeric center, producing a covalent-bound intermediate of the nucleophile with Walden inversion. Next, the intermediate undergoes inverting hydrolysis. The reaction of the inverting GH is similar but differs in the reagent undergoing inversion at the same site on the enzyme before it is released from the active center, which is the normal reaction of an inverting GH (9, 10). The mechanism was confirmed by analyzing the reaction of trehalase, which hydrolyzed an $\alpha$-1, $\alpha$-1 linkage with anomeric inversion (11). This mechanism was later named the “Hehre resynthesis-hydrolysis mechanism” (10).

In 1998, Withers and co-workers (12) reported that a mutant retaining enzyme, GH 1 $\beta$-glucosidase from Agrobacterium sp., with a mutation at its nucleophilic residue (Glu-358), catalyzed synthesis of various $\beta$-glucosides using $\alpha$-glucosyl fluoride as a donor and various p-nitrophenyl-$\beta$-glucosides as acceptors (12). They named this enzyme “glyco-synthase,” which catalyzed the synthesis of glycosides from the glycosyl fluoride of the opposite anomer catalyzed by a nucleophile-mutant retaining GH. To date, various retaining GHs have been converted into glycosynthases by substituting their nucleophilic residues (13–22).

Williams and Withers (10) commented that the glycosynthase technique was developed by mimicking the Hehre resynthesis-hydrolysis mechanism of the inverting GHs. However, no glycosynthase mutant derived from inverting GH has yet been reported. Because both the retaining and inverting GHs hydrolyze glycosides by two acidic catalytic residues as a general acid and a general base, we considered that removal of the general base from an inverting GH may result in production of a glycosynthase.

Recently, we found an interesting inverting GH that hydrolyzed xylooligosaccharides ($\geq X_3$) to release $X_1$ at their reducing end in the genome of Bacillus halodurans C-125 and named it reducing-end xylo-se-releasing exooligoxylanase (Rex, EC 3.2.1.156) (23). Structural analysis of Rex revealed the basis of the reducing-end exohydrolytic activity (24, 25). The catalytic residues were found to be Glu-70 as the general acid and Asp-263 as the general base (23–25).

We considered Rex to be a good model to analyze the Hehre resynthesis-hydrolysis mechanism as well as for the generation of a glycosynthase mutant due to its reaction specificity. Because it hydrolyzed xylooligosaccharides at the bond of the reducing end to form $X_1$ from the reducing end and $X_3$ from the non-reducing end, $\alpha$-xylobiosyl fluoride ($\alpha$-$X_3F$) and xylose are expected to be a suitable donor and acceptor, respectively. The strict exo-specificity is expected to prohibit both $\alpha$-$X_3F$ and $X_2$, the hydrolytic product of $\alpha$-$X_3F$, from acting as the acceptor. Thus, the donor and acceptor molecules could be treated independently during analyses of the Rex reaction. Here, we describe the first glycosynthase derived from an inverting GH as well as clear evidence for the Hehre resynthesis-hydrolysis mechanism using Rex.
EXPERIMENTAL PROCEDURES

Materials—Xylooligosaccharides (Xn; where n = degree of polymerization) were purchased from Megazyme (Wicklow, Ireland). α-X2F was synthesized by reaction of hexa-O-acetyl xylobiose with pyridinium poly(hydrogen fluoride) followed by O-deacetylation using sodium methoxide in methanol according to the standard procedure (26, 27). Other reagents were of analytical grade and were obtained from commercial sources.

DNA Manipulation—Recombinant DNA techniques and agarose gel electrophoresis were performed as described by Sambrook et al. (28). Plasmid DNA was prepared using a QIAprep Spin Plasmid kit (Qiagen, Hilden, Germany). Digestion by restriction enzymes was carried out in the appropriate buffer at concentrations of 1–10 units/mg of DNA for 0.5–16 h at 37 °C. Completion of the reaction was confirmed by agarose-gel electrophoresis. A QIAEX Agarose Gel Extraction kit (Qiagen) was used for extraction and purification of DNA from agarose gels.

Nucleotide Sequence Analysis—The nucleotide sequence was determined by the dideoxynucleotide chain termination method using an automated DNA sequencer (Model 310A; Applied Biosystems, Foster City, CA) with a dRhodamine Terminator kit (PerkinElmer Life Sciences). Sequence data were analyzed using GENETYX MAC software Version 11.0 (GENETYX Software Development Co. Ltd., Tokyo, Japan).

Construction of D263X Mutant Library—The gene encoding wild-type Rex from B. halodurans C-125 was constructed as reported previously (23). Saturation random mutagenesis at Asp-263 in Rex was performed using the PCR-overlap extension method (29). The following mutagenic oligonucleotide primer was used (the mismatched bases are underlined): 5'-CAC TTT TTT AGC NNN TCT TAT CGT GTG GCT-3' (N = A, T, G, and C). Next, the mutated gene encoding (D263X) was amplified by PCR using the forward primer, 5'-CCT TCC ATG GAG AAA ACG ACA GAA GGT GCA TTT-3' (containing an NcoI site; indicated in bold), and the reverse primer, 5'-GAA CTC GAG GTG TTC CTC TCT TGG CCC TCA G-3' (containing an XhoI site; indicated in bold). The amplified fragment was digested with the corresponding restriction enzymes. The digested fragment was ligated into pET28b (Novagen, Madison, WI) at the corresponding sites, generating the plasmid pET28b-D263X encoding D263X protein, with the His6 sequence added to its C-terminal end. pET28b-D263X was electroporated into Escherichia coli BL21GOLD(DE3) cells, and 120 positive colonies were selected.

Preparation of D263X Library—Each transformant was incubated in Luria broth (1 ml) containing 0.05 mg/ml kanamycin at 25 ºC using Overnight Express™ Autoinduction system 1 (Novagen) in accordance with the manufacturer’s instructions. The D263X proteins expressed were extracted from the wet cells using Bug Buster™ HT (Novagen). Subsequently, the extracts (0.6 ml) were loaded onto nickel nitrilotriacetic acid spin columns (Qiagen), and the enzyme was eluted with a stepwise increase in imidazole concentration (1, 10 mM; 2, 20 mM; 3, 250 mM) in 50 mM sodium phosphate buffer (pH 8.0) containing 0.3 M NaCl. In kinetic analysis, wild-type, D263C, and D263N were desalted using a PD-10 column (Amersham Biosciences). The purity of the proteins was checked by SDS-PAGE (30). Protein concentrations were determined from the absorbance at 280 nm, based on the theoretical molar absorption coefficients (106,210 M⁻¹ cm⁻¹) as described previously (31).

Analysis of the Reaction Products—The reaction products from α-X2F and X1 were separated by thin layer chromatography (TLC) on silica gel 60 F254 plates (5.0 × 7.5 cm; Merck) with a solvent system of acetonitrile/water (4:1, v/v). Sugars were detected by baking after dipping the plates in 5% sulfuric acid in methanol. When necessary, the

FIGURE 1. Hydrolytic mechanism of typical glycoside hydrolase. A, retaining enzyme. B, inverting enzyme. It should be noted that the protonations of the catalytic acidic residues of the inverting enzyme are reversed after the reaction.
Glycosynthase from an Inverting Glycoside Hydrolase

amounts of the products were quantified by high performance ion exchange chromatography on a CARBOPAC PA1 column (4 × 250 mm, Dionex, Sunnyvale, CA) equipped with a pulsed amperometric detector (DX-3, Dionex). Chromatography was performed with a linear gradient of 0–0.2 M sodium acetate in 0.1 M NaOH for 20 min at a flow rate of 1 ml/min.

Screening of Glycosynthase—Each D263X protein was incubated with the substrates (20 mM α-X2F and X3) in 0.1 M MOPS buffer (pH 7.0) for 15 h at 30 °C. The saccharides in the reaction solution were checked by TLC as described above.

Enzyme Assay—The enzymatic fluoride ion release reaction was performed in 0.1 M MOPS buffer (pH 7.0) containing 20 mM α-X2F and X1 at 30 °C. The reaction was monitored using a fluoride selective electrode (9609 BN, Thermo Orion, Beverly, MA) interfaced with a portable pH/ion-selective electrode meter model 290A+ (Thermo Orion). A fluoride ion standard curve was determined using sodium fluoride solution under the same reaction conditions without enzyme and substrates.

To determine the apparent kinetic parameters of the glycosynthase reaction, α-X2F and X3 were subjected to the fluoride ion release in 0.1 M MOPS buffer (pH 7.0) at 30 °C. The initial rates were measured as the increase in fluoride ions, as described above. The kinetic parameters were calculated by regressing the experimental data (substrate concentration range, 0.2–3 K_m^i) with the Michaelis-Menten equation by the curve-fitting method using Kaleidagraph™ Version 3.51 (Synergy Software, Reading, PA).

To determine the apparent kinetic parameters of X3 hydrolytic activity, X3 was subjected to hydrolysis in 0.1 M MOPS buffer (pH 7.0) at 30 °C. The initial rates were determined by measurement of the increase in xylose by high performance ion exchange chromatography, as described above. The kinetic parameters were calculated by regressing the experimental data (substrate concentration range, 0.2–3 K_m^i) with the Michaelis-Menten equation by the curve-fitting method as described above.

Time Course of Enzymatic Reaction toward α-X2F and X3—The enzymatic reactions were carried out with wild-type, D263C, and D263N in 0.1 M MOPS buffer (pH 7.0) at 30 °C. At appropriate reaction times, aliquots of the reaction solution were withdrawn and diluted with distilled water (1:100). The solutions were subjected immediately to high performance ion exchange chromatography and analyzed as described above. Under the standard conditions for xylooligosaccharide separation, peaks of X1 and α-X2F overlapped at the same retention time, and therefore, only X3 and X3 were quantified in the reaction. In the enzymatic reaction, fluoride ions in the reaction solution were also detected using a fluoride electrode as described above.

RESULTS

Activity of the Wild-type Rex on α-X2F—We examined the reaction of wild-type Rex on α-X2F (20 mM) in both the presence and absence of X1 (20 mM) by TLC (Fig. 2). No product was detected in the absence of X1, even at extended incubation times. In contrast, a decrease in α-X2F with a concomitant increase in X3 was observed in the presence of X1. In addition, an increase in F− concentration was detected during the reaction. These results suggested that α-X2F was hydrolyzed only in the presence of X1, indicating that the reaction was not simple hydrolysis of α-X2F.

Screening of Glycosynthase by Saturation Random Mutagenesis—From the D263X mutant library constructed by saturation random mutagenesis, 120 colonies were obtained, and the mutant enzymes were purified by His-tag affinity column chromatography. During the screening of α-X2F-consuming activity on TLC, 71 of the 120 proteins exhibited activity. Other mutants did not consume α-X2F. Their nucleotide sequences were analyzed, and nine types of D263X mutant were found in addition to the wild type. The numbers of proteins obtained were as follows: Asp (wild-type), 15; Gly, 22; Ala, 12; Val, 8; Thr, 5; Leu, 3; Asn, 2; Cys, 2; Pro, 1; and Ser, 1. The reactions catalyzed by each mutant were analyzed. As summarized in Table 1, D263C exhibited the greatest production of X3, and D263N exhibited the highest activities of α-X2F consumption and production of X3 among the mutants. Thus, we selected D263C and D263N as targets for further analysis of glycosynthase properties.

Time Courses of the Reaction by the Wild-type and Glycosynthase Mutants—Fig. 3 shows the time courses of α-X2F and X3 reaction by the enzymes. In the wild-type reaction, F− and X2 concentrations increased in parallel, but X3 was not detected. On the other hand, X3 was detected in the reactions catalyzed by the mutants. In the case of D263N, X2 concentration was higher than X3 in the reaction mixture, indicating that the hydrolytic reaction was higher than the transfer reaction. Therefore, the X3 level decreased gradually with reaction time. In contrast, X3 concentration was higher than X3 in the reaction with D263C, indicating that the transfer activity was higher than the hydrolytic activity. Judging from the product concentration ratio between X1 and X3 at 3.2 h, the glycosynthase activity of D263C was much higher than that of D263N (X3/X2, D263C = 1.8, D263N = 0.06). The resulting X3 and X3 were β-1,4-linked xylooligosaccharides evidenced by comparing their 1H NMR spectra with those of the authentic X3 and X3, respectively (data not shown).

TABLE 1

| X3 | X2 | α-X2F remaining |
|----|----|-----------------|
| No enzyme | - | - | +++++ |
| Gly | + | + | + |
| Ala | + | + | +++++ |
| Val | + | + | +++++ |
| Thr | + | + | + |
| Leu | + | + | +|
| Cys | +++++ | + | - |
| Asn | + | + | + |
| Pro | + | + | + |
| Ser | + | + | +++++ |
| Asp (wild type) | - | ++++++ | - |
The enzymatic reaction was carried out in 0.1 M MOPS buffer (pH 7.0) at 30 °C. In α-X_F and X_1 reactions, the other substrate was fixed at 10 mM. Other conditions are described under “Experimental Procedures.” The reaction rates were determined by quantifying fluoride ions.

### TABLE 2

| Enzyme   | k_cat (s^{-1}) | k_m (mM) | k_cat/k_m (s^{-1} mM^{-1}) |
|----------|----------------|----------|---------------------------|
| Wild-type| ND*            | ND*      | 0.30 ± 0.01               |
| D263C    | 0.45 ± 0.10    | 4.2 ± 0.4 | 0.11 ± 0.01               |
| D263N    | 0.60 ± 0.05    | 10.5 ± 1.8 | 0.06 ± 0.01               |

* ND, not determined due to the linear relationship of the [S]-v curve.

### TABLE 3

| Enzyme   | k_cat (s^{-1}) | k_m (mM) | k_cat/k_m (s^{-1} mM^{-1}) |
|----------|----------------|----------|---------------------------|
| Wild-type| 5.04 ± 0.24    | 6.6 ± 1.1 | 0.77 ± 0.01               |
| D263C    | 0.32 ± 0.02    | 1.0 ± 0.2 | 0.31 ± 0.05               |
| D263N    | 0.35 ± 0.01    | 1.1 ± 0.1 | 0.30 ± 0.03               |

**DISCUSSION**

This is the first report of a glycosynthase derived from an inverting glucoside hydrolase. All the glycosynthases reported previously were derived from retaining enzymes. As reported by Withers and co-workers (10, 12), who first demonstrated the glycosynthase technique, the reaction of glycosynthase mimics the reaction of inverting enzymes, in which the glycosyl fluoride of the opposite anomer (e.g. α-glycosyl fluoride by β-glycoside-hydrolyzing enzyme) is hydrolyzed in the presence of the acceptor molecule. Several inverting enzymes have been reported to hydrolyze the opposite anomer of glycosyl fluoride (6, 11, 32–34). The mechanism of the reaction, illustrated in Fig. 4, is called the Hehre resynthesis-hydrolysis mechanism (9, 10), because the reaction was found and investigated by Hehre’s group. In this mechanism, the wrong glycosyl fluoride is transferred to the hydroxyl group of the acceptor with the Walden inversion releasing the F⁻ to form the correct O glycosyl linkage followed by hydrolysis of the newly formed glycoside before it is released from the active center with anomeric inversion.

The Hehre resynthesis-hydrolysis mechanism is often difficult to
detect because many enzymes recognize glycosyl fluoride as not only the donor but also as the acceptor (7–10). Reliable experimental evidence for the mechanism was reported for trehalase using β-glucosyl fluoride as the donor and glucose or xylose as the acceptor (11, 33). However, some uncertainty remains because the donor, β-glucosyl fluoride, is hydrolyzed into the acceptor, glucose, making the reaction complicated.

We consider Rex to be the suitable tool to examine the mechanism. Because Rex hydrolyzes X₃ to release X₁ from the reducing end, it is expected to utilize X₂F as the donor and X₁ as the acceptor. It should be mentioned that the hydrolytic product of α-X₂F, X₂, will never act as the acceptor molecule due to the reducing-end exo-specificity. Thus, the donor and acceptor are completely independent during the reaction. Our results clearly confirmed the Hehre resynthesis-hydrolysis mechanism because the hydrolysis of α-X₂F was observed only in the presence of X₁. The kinetic parameters in the mechanism were given. No X₃ was detected during the reaction. Most of the resulting X₃ was supposed to be hydrolyzed into X₁ and X₂ before it escaped from the active center because X₃ was generated at the very position where it was hydrolyzed.

Next, we attempted to obtain a glycosynthase mutant of Rex using the saturation mutagenesis technique at the catalytic base, Asp-263. Nine amino acid residues showed some glycosynthase activity. Among them, D263C and D263N mutants exhibited high levels of glycosynthase activity. The time courses of the reaction indicated that D263C was the best mutant to produce X₃. A Cys mutant has not been found as the best mutation in glycosynthases derived from retaining enzymes. For example, in the case of Agrobacterium sp. β-glucosidase belonging to GH 1, the Cys mutant was 100-fold less active than the Gly mutant. The Gly mutants give the highest glycosynthase activity of all the retaining glycosynthase reported (14, 18, 19, 35). The time course of the reaction of the glycosynthase mutants showed that X₂, the hydrolytic product, was formed from the early stages of the reaction, indicating that a portion of

FIGURE 4. Hehre resynthesis-hydrolysis mechanism for hydrolysis of the wrong glycosylfluoride by inverting enzymes. The protonations of the catalytic acidic residues are reversed at the initial stage, because the initial reaction in the mechanism mimics the reverse hydrolysis of the inverting enzyme shown in Fig. 1B.

FIGURE 5. Reaction mechanism of glycosynthase derived from inverting and retaining GHs. A, Rex. B, Agrobacterium sp. β-glucosidase.
X₃ formed by condensation was hydrolyzed before it was released from the enzyme.

The $F^-$-releasing activities from $\alpha$-$X₃F$ and the hydrolytic activities on $X₃$ were measured and compared with those of the wild type. Comparing $k_{cat}/K_{m}$, the $F^-$-releasing activities of both the E263C and E263N mutants were similar and were slightly less than half of that of the parent. In contrast, the hydrolytic activity of the E263C mutant was 10-fold lower than that of the E263N mutant. The difference in hydrolytic activity makes the Cys mutant a better glycosynthase than the Asn mutant. However, the Cys mutant still retains 0.02% hydrolytic activity of the parent.

The relationship between the $F^-$-releasing activity and the hydrolytic activity of Rex is completely different from those of the retaining enzymes (Fig. 5). In the case of retaining enzymes, the enzyme possesses $F^-$-releasing activity (10). On the other hand, mutants of the retaining enzymes retain only negligible hydrolytic activities (36). Mutation of the nucleophile residue completely removes the nucleophilic reagent in the retaining enzymes, because the nucleophile residue directly attacks the C1 of the hydrolyzed glycoside. In contrast, in the case of the inverting enzymes, the water molecule activated by the base residue attacks the C1 of the hydrolyzed glycoside. Thus, the mutation of the base residue cannot remove the hydrolytic activity completely because the water molecule retains some activity without the aid of the catalytic residue.

For the above reasons, the mechanisms of acquisition of glycosynthase activity from the retaining and inverting enzymes are different. For the retaining enzymes, it is important to acquire $F^-$-releasing activity by mutation. In the case of inverting enzymes, it is important to minimize the decrease in $F^-$-releasing activity while maximizing the decrease in hydrolytic activity. In conclusion, we successfully converted an inverting enzyme to a glycosynthase. Our research expands the possibility of deriving glycosynthases from not only retaining enzymes but also from inverting enzymes.

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