Glycosynthase Activity of *Bacillus licheniformis* 1,3-1,4-β-Glucanase Mutants: Specificity, Kinetics, and Mechanism†

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ABSTRACT: Glycosynthases are engineered retaining glycosidases devoid of hydrolase activity that efficiently catalyze transglycosylation reactions. The mechanism of the glycosynthase reaction is probed with the E134A mutant of *Bacillus licheniformis* 1,3-1,4-β-glucanase. This *endo*-glycosynthase is regiospecific for formation of a β-1,4-glycosidic bond with α-glycosyl fluoride donors (laminaribiosyl as the minimal donor) and oligosaccharide acceptors containing glucose or xylose on the nonreducing end (aryl monosaccharides or oligosaccharides). The pH dependence of the glycosynthase activity reflects general base catalysis with a kinetic pK_a of 5.2 ± 0.1. Kinetics of enzyme inactivation by a water-soluble carbodiimide (EDC) are consistent with modification of an active site carboxylate group with a pK_a of 5.3 ± 0.2. The general base is Glu138 (the residue acting as the general acid−base in the parental wild-type enzyme) as probed by preparing the double mutant E134A/E138A. It is devoid of glycosynthase activity, but use of sodium azide as an acceptor not requiring general base catalysis yielded a β-glycosyl azide product. The pK_a of Glu138 (kinetic pK_a on k_{cat}/K_M and pK_a of EDC inactivation) for the E134A glycosynthase has dropped 1.8 pH units compared to the pK_a values of the wild type, enabling the same residue to act as a general base in the glycosynthase enzyme. Kinetic parameters of the E134A glycosynthase-catalyzed condensation between Glcβ4Glcβ3GlcαF (**2**) as a donor and Glcβ4Glcβ-pNP (**15**) as an acceptor are as follows: k_{cat} = 1.7 s^{-1}, K_M(acceptor) = 11 mM, and K_M(donor) < 0.3 mM. Donor self-condensation and elongation reactions are kinetically evaluated to establish the conditions for preparative use of the glycosynthase reaction in oligosaccharide synthesis. Yields are 70−90% with aryl monosaccharide and cellobioside acceptors, but 25−55% with laminaribiosides, the lower yields (and lower initial rates) due to competitive inhibition of the β-1,3-linked disaccharide acceptor for the donor subsites of the enzyme.

Retaining glycosidases are common biocatalysts used in chemoenzymatic oligosaccharide synthesis under conditions that favor reversal of their hydrolytic reactions. This is accomplished either by displacing the equilibrium with a large excess of acceptor (thermodynamically controlled synthesis) or by using activated glycosyl donors (kinetically controlled transglycosylation) (*1*−*3*). Scheme 1 illustrates the well-established hydrolysis mechanism of a retaining glycosidase, in which two essential active site carboxyl groups provide a general acid−base and nucleophilic catalysis in a double-displacement mechanism (*4*−*7*). Under proper conditions, kinetically controlled transglycosylation takes advantage of sugar donors with excellent leaving groups, such as glycosyl fluorides and aryl glycosides, to increase the concentration of the glycosyl−enzyme intermediate which can then be intercepted by an acceptor other than water. Even after careful optimization of reaction conditions and solvent composition, condensation or transglycosylation yields rarely exceed 50% because of the hydrolase activity of the enzyme on the newly formed glycosidic linkage.

Recent advances for overcoming this limitation came up with the glycosynthase concept: a hydrolytically inactive glycosidase in which the catalytic nucleophile has been replaced by a smaller non-nucleophilic residue catalyzes efficiently the transglycosylation between an activated glycosyl fluoride donor (which has an anomeric configuration that is the opposite of that of the normal substrate of the parental wild-type glycosidase) and an acceptor (*8*, *9*). As shown in Scheme 2, an α-glycosyl fluoride donor mimics the glycosyl−enzyme intermediate (Scheme 1) in the active site of the nucleophile-less mutant of a β-glycosidase, and then is able to transfer the glycone to an acceptor that binds in the acceptor subsites of the enzyme. The product that is formed is unable to be hydrolyzed by the mutant enzyme, so transglycosylation yields are very high. The first glycosynthase was described on the *exo*-enzyme β-glucosidase from *Agrobacterium faecalis* (*10*), and subsequently applied on the *endo*-acting 1,3-1,4-β-glucanase[1] from *Bacillus licheniformis* in our group (*11*). Since then, few other

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[1] Abbreviations: 1,3-1,4-β-glucanase, 1,3-1,4-β-D-glucan 4-glucanohydrolase (EC 3.2.1.73); EDC, 1-ethyl 3-[3-(dimethylamino)propyl]-carbodiimide; MU, methylumbelliferyl; pNP, *p*-nitrophenyl; TNM, tetranitromethane.

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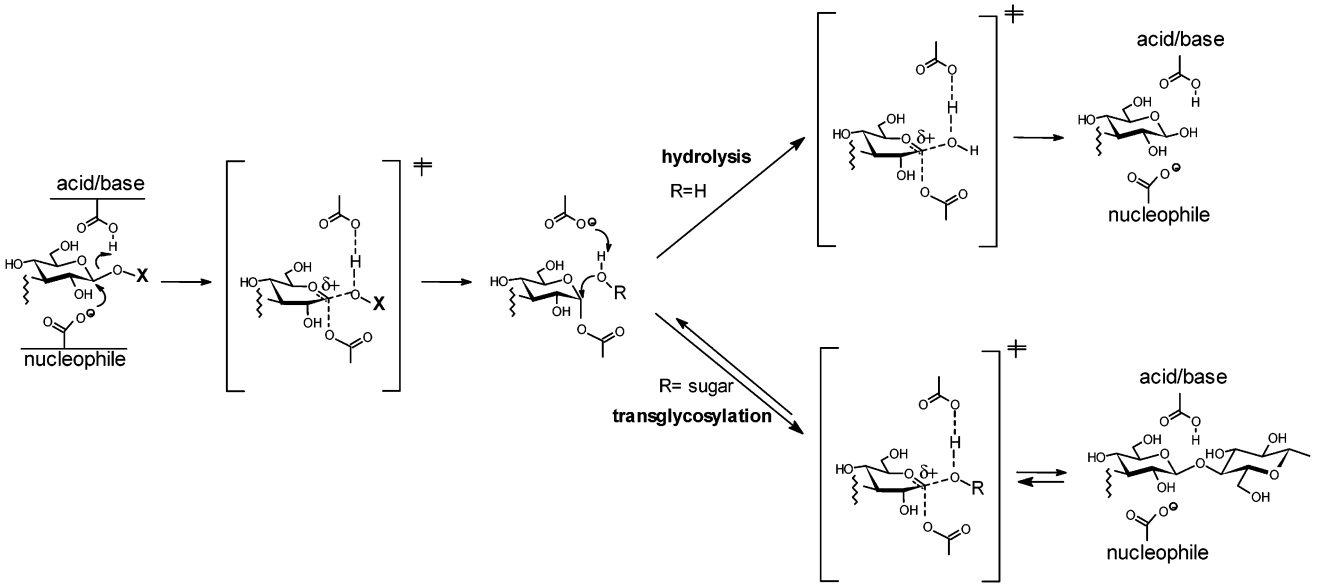
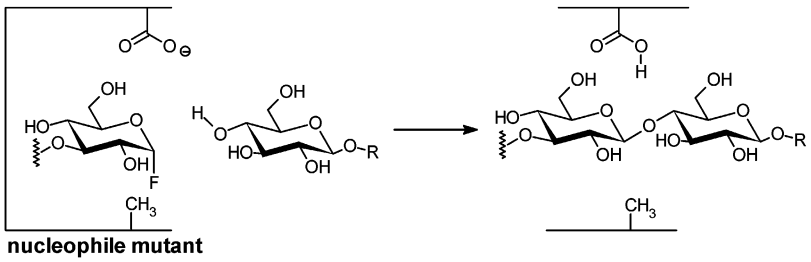
Scheme 1: Double-Displacement Mechanism of a Retaining β-Glycosidase[a]



[a] Normal hydrolytic reaction in the top branch and transglycosylation reaction with a sugar acceptor in the bottom branch.

Scheme 2: Glycosynthase Mechanism[a]



[a] Nucleophile-less mutant of a retaining β-glycosidase with an α-glycosyl fluoride donor and a glycoside acceptor to yield a condensation (transglycosylation) product.

glycosynthases have been constructed, derived from both *exo*-glycosidases [β-glycosidase from *Solfolobus solfataricus* (*12*), β-mannosidase from *Cellulomonas fimi* (*13*), β-galactosidase from *Escherichia coli* (*14*), and α-glucosidase from *Schizosaccharomyces pombe* (*15*)] and *endo*-glycosidases [cellulase from *Humicola insolens* (*16*), 1,3-β-glucanase from *Hordeum vulgare* (*17*), and mannanase from *Camponotus japonicus* (*18*)].

We describe here the detailed characterization of the first reported *endo*-glycosynthase, which was derived from *B. licheniformis* 1,3-1,4-β-glucanase, with regard to specificity, mechanism, and synthetic applications. *Bacillus* 1,3-1,4-β-glucanases have been widely studied in our laboratory through mechanistic, reactivity, specificity, and structure−function analyses (*19*). These enzymes are family 16 glycosyl hydrolases (retaining) (*20*) with molecular masses of 25−30 kDa, with a monomeric jellyroll β-sandwich structure that possesses an open binding site cleft composed of four glucosyl-binding subsites on the nonreducing end from the scissile glycosidic bond, and two subsites on the reducing end. The catalytic residues are two glutamates, namely, Glu138 as the general acid−base and Glu134 as the catalytic nucleophile in the *B. licheniformis* enzyme (*21*). The single-Ala mutants in both residues are hydrolytically inactive enzymes, but the activity is rescued by addition of azide as an exogenous nucleophile to yield glycosyl−azide adducts (the α-anomer for the nucleophile mutant E134A and the β-anomer for the general acid−base mutant E138A). A third carboxyl residue, Asp136, is also important for catalysis, contributing to the pK_a modulation of the catalytic residues (manuscript submitted for publication); the H-bond between Asp136 and the nucleophile Glu134 in the free enzyme (*22*, *23*) is disrupted in the covalent glycosyl−enzyme intermediate, where Asp136 is now within hydrogen bonding distance of the general acid Glu138. On the other hand, the nucleophile mutant E134A 1,3-1,4-β-glucanase from *B. licheniformis* proved to be an efficient glycosynthase able to catalyze the condensation of α-laminaribiosyl fluoride with glucosides and cello- and laminaribiosides in excellent yields (*11*). In combination with another *endo*-glycosynthase, the E197A cellulase Cel7B, tandem glycosylations have been performed to prepare complex mixed linkage oligosaccharides (*24*). Here, detailed characterization of the E134A glycosynthase derived from *B. licheniformis* 1,3-1,4-β-glucanase is reported.

MATERIALS AND METHODS

Substrates. The acceptor substrates 4-nitrophenyl β-glucoside (**5**), methyl β-glucoside (**6**), 4-nitrophenyl β-xyloside (**8**), 4-nitrophenyl 2-acetamido-2-deoxy-β-glucoside (**10**), 4-methylumbelliferyl β-glucuronic acid (**11**), methyl α-glucoside (**13**), and maltose (**20**) were from Fluka. 4-Methylumbelliferyl β-xyloside (**7**) was from Lancaster, and 4-nitro-

phenyl β-mannoside (**9**), 4-nitrophenyl β-galactoside (**12**), sucrose (**19**), and isomaltose (**21**) were from Sigma. 4-Methylumbelliferyl β-glucoside (**4**) and 4-methylumbelliferyl β-laminaribioside (**17**) were prepared as previously described (*25*); methyl β-laminaribioside (**18**) was prepared as described in ref *26*, and 4-methylumbelliferyl β-cellobioside (**14**), 4-nitrophenyl β-cellobioside (**15**), and methyl β-cellobioside (**16**) were prepared as described in ref *27*.

The glycosyl donor α-laminaribiosyl fluoride (**1**) was prepared as previously reported (*11*) by reaction of laminaribiose peracetate with hydrogen fluoride in pyridine (70%) followed by de-O-acetylation with sodium methoxide in methanol. Likewise, the tri- and tetrasaccharide donors 3-*O*-β-cellobiosyl-α-D-glucosyl fluoride (**2**) and 3-*O*-β-cellotriosyl-α-D-glucosyl fluoride (**3**) were obtained with the same procedure starting from the corresponding peracetylated saccharides derived from enzymatic degradation of barley β-glucan by 1,3-1,4-β-glucanase from *B. licheniformis* (*28*). Spectral data of new donors **2** and **3** are given in the Supporting Information.

Substrates for assaying the hydrolase activity of 1,3-1,4-β-glucanases were obtained as previously reported: 4-methylumbelliferyl 3-*O*-β-cellobiosyl-β-D-glucopyranoside (**28**) (*25*, *28*) and 2,4-dinitrophenyl 3-*O*-β-cellobiosyl-β-D-glucopyranoside (**29**) (*29*).

Enzymes. Wild-type and E134A mutant 1,3-1,4-β-glucanases from *B. licheniformis* were expressed and purified as previously described (*21*). The double mutant E134A/E138A was prepared by site-directed mutagenesis by PCR using the full double-stranded plasmid DNA template method (*30*). Plasmid pD6-2::E134A with the coding sequence of the single mutant E134A 1,3-1,4-β-glucanase in a pUC119 vector (expression construct as described in ref *21*) was used as a template for the PCR using two oligonucleotide primers containing the E138A mutation: 5′-GCAATCGACATCGCGTTTCTAGGAAAAG-3′ and the complementary oligonucleotide 5′-CTTTTCCTAGAAACGCGATGTCGATTGC-3′. After the PCR in which the complete vector was amplified, the reaction mixture was directly used to transform TG1 *E. coli* cells. The gene of a selected clone was sequenced in full. BL21-SI *E. coli* cells were transformed with the mutagenized plasmid for protein expression following the same basic procedure described for the wild-type and E134A mutant proteins (*21*).

All three enzymes were lyophilized for storage (−20 °C) and dissolved in the reaction buffer prior to being used. The purity of the enzyme preparations was higher that 95% as judged by SDS−polyacrylamide gel electrophoresis according to the method of Laemmli (*31*). Enzyme concentrations were determined by UV spectrophotometry using an ϵ_{280} of 14.5 mg mL^{-1} cm^{-1} (3.55×10^5 M^{-1} cm^{-1}) (*32*).

Kinetics of Hydrolase Activity. Kinetics with substrates 4-methylumbelliferyl 3-*O*-β-cellobiosyl-β-D-glucopyranoside (**28**) and 2,4-dinitrophenyl 3-*O*-β-cellobiosyl-β-D-glucopyranoside (**29**) were performed by following changes in UV absorbance due to the release of 4-methylumbelliferone or 2,4-dinitrophenol using matched 1 cm path length cells in a Varian Cary 4 spectrophotometer with a Peltier temperature control system which kept the cells at 35 °C. Rates of the enzyme-catalyzed hydrolyses were determined by incubating the substrate at the appropriate concentration in citrate−phosphate buffer [6.5 mM citric acid, 87 mM Na_2HPO_4 (pH 7.2), and 0.1 mM $CaCl_2$] for 5 min in the thermostated cell holder, followed by addition of the corresponding enzyme and monitoring of the absorbance change at 365 nm for substrate **28** [$\Delta\epsilon = 5440$ M^{-1} cm^{-1} (*33*)] or 425 nm for substrate **29** [$\Delta\epsilon = 6134$ M^{-1} cm^{-1} (*29*)].

Kinetics of Glycosynthase Reactions. (1) HPLC Monitoring. The glycosyl donor and acceptor were dissolved in maleate buffer (50 mM, pH 7.0) containing $CaCl_2$ (0.1 mM), and the solution was preincubated at 35 °C for 5 min. Reactions were initiated by addition of the E134A mutant 1,3-1,4-β-glucanase (final volume of 0.3 mL) and the mixtures kept at 35 °C. Aliquots were taken at different time intervals and diluted 1:10 in deionized H_2O for HPLC analysis [NovaPak C18 (4 μm, 3.9 mm × 150 mm) column (Waters), rate of 1 mL/min, 16−18% MeOH in H_2O, UV detector at 316 nm (λ)]. Concentrations in the different experiments are indicated in Tables 1−3. Initial rates (v_0) were calculated from the linear portion of the progress curves (normalized area vs time) and expressed as v_0/[E] in inverse seconds. Chromatographic peaks were identified by co-injection with independent standards. For pH studies, citrate−phosphate buffer (50 mM each), 0.1 mM $CaCl_2$, and a constant ionic strength (0.5 M) with added KCl were used in the pH range of 5.0−9.0.

(2) Fluoride Selective Electrode Monitoring. Kinetics of fluoride release for spontaneous hydrolysis of the α-glycosyl fluoride donor, enzyme-catalyzed self-condensation of the donor, and transglycosylation reactions were monitored with a fluoride selective electrode (Sentek) interfaced with a CiberScan Bench pH/ion meter (Eutech Instruments). All reactions were performed in 50 mM maleate buffer (pH 7.0) and 0.1 mM $CaCl_2$ containing the substrates and enzyme as appropriate, in a final volume of 0.5 mL with stirring in a thermostated bath at 35 °C. Sodium fluoride standards for calibration curves were prepared under the same experimental conditions [50 mM maleate buffer (pH 7.0), 0.1 mM $CaCl_2$, and 35 °C] with or without enzyme and acceptor depending on the process being monitored.

In either case (1 or 2), initial velocities were fit to a normal Michaelis−Menten equation when saturation was observed, or to a substrate inhibition model (eq 1) when a maximal rate followed by a decrease at a high substrate concentration was obtained.

$$v_0 = \frac{k_{cat}[E]_0[S]}{K_M + [S] + \frac{[S]^2}{K_I}} \quad (1)$$

Preparative Glycosynthase Reactions. α-Laminaribiosyl fluoride (**1**) (30 mg, 0.09 mmol, 1 equiv), acceptor (5 equiv), and E134A 1,3-1,4-β-glucanase (0.5−1 mg) were dissolved in phosphate buffer (100 mM), $CaCl_2$ (0.1 mM) (pH 7.0), and the solution (total volume of 1.5 mL) was incubated at 35 °C for 1−2 days. Reactions were monitored by TLC (silica gel 60 F254 plates, Merck, eluted with a 8:2 or 7:3 CH_3CN/H_2O mixture). Workup and isolation of the condensation product were performed by two different procedures.

In procedure A (for reactions with monosaccharide acceptors **4** and **8**), the reaction mixture was directly loaded into a reverse-phase Lichoprep RP-18 Lobar-A column

(Merck) and eluted with a H_2O/MeOH gradient from 0 to 15% (v/v). Unreacted acceptor eluted first, followed by the condensation product. It was freeze-dried and lyophilized for storage.

In procedure B (for reactions with disaccharide acceptors **14** and **16**−**18**), the reaction mixture was lyophilized, and the solid was treated with acetic anhydride in pyridine (6 mL, 1:1, v/v) and catalytic amounts of (*N*,*N*-dimethylamino)-pyridine for 12 h at room temperature. After the usual workup (pouring on ice and water, extraction with $CHCl_3$, drying the organic phase with anhydrous $MgSO_4$, and solvent evaporation), the reaction mixture was purified by flash chromatography [2:1 (v/v) ethyl acetate/petroleum ether mixture]. Typically, three fractions were eluted: per-O-acetylated acceptor (in excess) first, the per-O-acetylated condensation product (30−90% yields from donor) second, and a mixture of acetylated higher oligomers as the minor fraction third. Spectral data (MS and ^{1}H and ^{13}C NMR) of the per-O-acetylated condensation products are given in the Supporting Information.

Per-O-acetylated oligosaccharides (70 mg) were treated with freshly prepared sodium methoxide (1 M in methanol, 0.3−1 mL) in methanol (30−50 mL) and stirred at room temperature for 5 h. In some cases, the de-O-acetylated oligosaccharide precipitated partially, the solid being filtered and washed with cold methanol. The reaction solution was neutralized with Amberlite IR 120 H^+ resin (Fluka) and filtered, and the solution was freeze-dried and lyophilized to afford the final condensation product in quantitative yield.

Spectral data (MS and ^{1}H and ^{13}C NMR) for condensation products obtained with acceptors **4**, **8**, **14**, and **16**−**18** are given in the Supporting Information.

Self-condensation of α-laminaribiosyl fluoride (**1**) yielded a mixture of oligomers. NMR data are given in the Supporting Information. Further characterization of higher oligomers will be reported elsewhere. Condensation products of α-laminaribiosyl fluoride (**1**) with acceptors Xyl-MU (**7**) and Glcβ4Glc-pNP (**15**) were not characterized [only analytical reactions for measuring initial rates (Table 3)]. Preparative reactions and product characterizations were performed with the homologous acceptors Xyl-pNP (**8**) and GlcβGlc-MU (**14**), respectively (see above).

Enzyme Inactivation by EDC. E134A 1,3-1,4-β-glucanase was preincubated in 20 mM MES/20 mM HEPES/40 mM 4-hydroxy-1-methylpiperidine (pH 5.6) ternary buffer (*34*) at 25 °C for 5 min. A solution of EDC (final concentration of 0−120 mM) was added in the presence or absence of glycine methyl ester (final concentration of 0−1.2 M), and the reaction mixtures (26 μM in enzyme) were kept at 25 °C for 1−2 h. At regular time intervals, 20 μL aliquots were withdrawn and added to 30 μL of sodium acetate (167 mM, pH 4.7, 0 °C) to quench the excess of EDC reagent. The remaining glycosynthase activity of the enzyme was determined by assessing the glycosynthase-catalyzed self-condensation of donor Gluβ4Gluβ3GluαF (**2**, 2 mM) as described above (F^- release in 50 mM maleate buffer and 0.1 mM $CaCl_2$ at pH 7.0 and 35 °C). Activity was corrected for nonenzymatic spontaneous hydrolysis of the fluoride donor and expressed as a percentage of the initial enzyme activity (no EDC).

The pH dependence of enzyme inactivation by EDC was investigated by incubation of the E134A enzyme (26 μM) with EDC (120 mM) in the same ternary buffer (20 mM MES, 20 mM HEPES, and 40 mM 4-hydroxy-1-methylpiperidine) at pH values ranging from 4.0 to 9.0 (ionic strength is constant with this buffer over the pH range). Remaining glycosynthase activity was determined as described above.

To analyze the reversibility of enzyme inactivation, a sample of EDC-modified enzyme [25 μM E134A enzyme and 120 mM EDC (pH 5.8) for 2 h at 25 °C and then quenched with 100 mM sodium acetate (pH 4.7)] was extensively dialyzed against 100 mM sodium acetate (pH 4.8) in a Microkon spinning ultrafiltration unit (Amicon, Millipore), and the residual glycosynthase activity was determined as described above. A control enzyme sample (not treated with EDC) was subjected to the same procedure.

Nitration of Tyr Residues by TNM. Nitration of Tyr residues of E134A 1,3-1,4-β-glucanase was performed according to the protocol of Sokolovsky *et al.* (*35*). A solution of tetranitromethane (1.18 mM) and enzyme (22.8 μM) in Tris-HCl buffer (100 mM, pH 8.0) was incubated at 25 °C for 10 h. The number of modified Tyr residues was monitored spectrophotometrically using an ϵ_{428} of 4100 M^{-1} cm^{-1} for 3-nitrotyrosine. Enzyme activity was monitored in a parallel reaction under the same experimental conditions. At appropriate time intervals, aliquots (20 μL) were withdrawn and added to sodium acetate (30 μL, 167 mM, pH 4.7, 0 °C). The remaining glycosynthase activity was determined as described above, and expressed as a percentage relative to that of a control reaction which followed the same protocol with no TNM.

Chemical Rescue of E134A/E138A Mutant 1,3-1,4-β-Glucanase. ^{1}H NMR monitoring was performed following a modification of the procedure reported in Malet *et al.* (*36*). ^{1}H NMR spectra were recorded on a Varian Gemini 300 spectrometer. Experiments were carried out at 35 °C in buffered D_2O (pD 7.2, 50 mM deuterated phosphate buffer and 0.1 mM $CaCl_2$), and spectra were recorded at 1−2 h intervals for up to 74 h. Substrates, enzymes, and sodium azide concentrations in the different experiments were as follows: (a) 34 μM E134A, 11.8 mM Glcβ4Glcβ3Glcβ-2,4DNP, and 2 M NaN_3 and (b) 34 μM E134A/E138A, 11.8 mM Glcβ4Glcβ3Glcβ-2,4DNP, and 2 M NaN_3 for substrate Glcβ4Glcβ3Glcβ-2,4DNP (**29**) and (a) 19 mM Glcβ3GlcαF (**1**) and 1 M NaN_3, (b) 34 μM E134A/E138A and 19 mM Glcβ3GlcαF (**1**), (c) 34 μM E134A/E138A, 19 mM Glcβ3GlcαF (**1**), and 2 M NaN_3, (d) 15 mM Glcβ4Glcβ3GlcαF (**2**) and 2 M NaN_3, (e) 34 μM E134A/E138A and 15 mM Glcβ4Glcβ3GlcαF (**2**), (f) 34 μM E134A/E138A, 15 mM Glcβ4Glcβ3GlcαF (**2**), and 2 M NaN_3 for substrate α-glycosyl fluorides.

Assignments of the NMR signals [δ (parts per million) and J (hertz)] are given in the legends of Figures 7 and 8.

RESULTS

Donor Specificity. On the basis of the substrate specificity of the wild-type enzyme (*33*), the minimal donor for the glycosynthase reaction of the E134A 1,3-1,4-β-glucanase is the α-laminaribiosyl fluoride (**1**). A set of donors with an increasing degree of oligomerization (**1**−**3**, Scheme 3) were assayed using 4-methylumbelliferyl β-cellobioside (**14**) as

Scheme 3: Glycosynthase Reactions of E134A 1,3-1,4-β-Glucanase (*B. licheniformis*) with α-Glycosyl Fluoride Donors **1**−**3** and a 4-Methylumbelliferyl β-Cellobioside (**14**) Acceptor

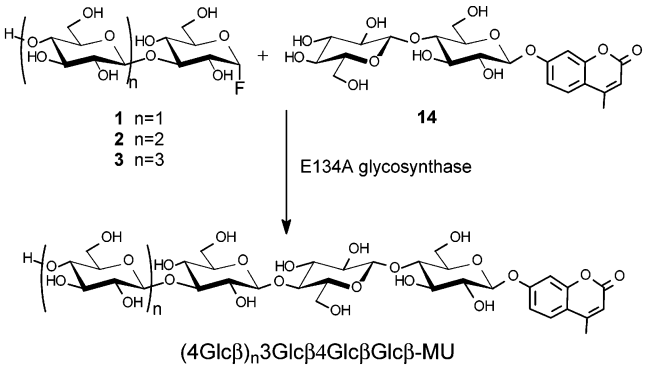


Table 1: Kinetic Parameters of the E134A Glycosynthase-Catalyzed Condensation of Donors **1**−**3** and the Acceptor 4-Methylumbelliferyl β-Cellobioside (**14**)[a]

donor[b]	K_M^{app} (mM)	k_{cat} (s^{-1})	k_{cat}/K_M^{app} (mM^{-1} s^{-1})
1 Glcβ3GlcαF	1.36 ± 0.18	0.040 ± 0.003	0.030 ± 0.006
2 Glcβ4Glcβ3GlcαF	0.34 ± 0.04	0.91 ± 0.03	2.65 ± 0.42
3 Glcβ4Glcβ4Glcβ3GlcαF	0.15 ± 0.03	1.35 ± 0.09	8.98 ± 2.24

[a] Conditions: 50 mM maleate buffer, pH 7.0, 0.1 mM $CaCl_2$, 35 °C, [E134A] = 30−300 μM, and [acceptor Glcβ4Glc-MU] = 7.8 mM. [b] α-D-Glucosyl fluoride does not react as a glycosyl donor.

an acceptor. Reactions were performed in maleate buffer at pH 7.0 and 35 °C, conditions previously found to be optimal with the model reaction Glcβ3GlcαF (**1**) + Glc-MU (**4**) (*11*). Kinetics of the glycosynthase reactions were determined by HPLC monitoring where the initial rates of transglycosylation at different concentrations of donor are calculated from the relative areas of transglycosylation product at initial reaction times (<10% conversion, linear progress curve). Kinetic parameters for donors **1**−**3** are summarized in Table 1. Normal Michaelis−Menten behavior was observed for the di- and trisaccharide donors **1** and **2**, whereas some substrate inhibition was apparent with the larger tetrasaccharide donor **3** (Figure 1).

Acceptor Specificity. The specificity of the acceptor subsites of the enzyme (+1 and +2) was studied with series of mono- and disaccharide acceptors. First, the relative yields in transglycosylation products after 24 h reactions using α-laminaribiosyl fluoride (**1**) as the donor and the different acceptors (**4**−**20**) in a 1:2 molar ratio (3 mM donor, 6 mM acceptor, 3 μM enzyme, pH 7.0, and 35 °C) were determined by HPLC in an effort to evaluate the relative reactivities of the acceptors under the same experimental conditions. Results are presented in Table 2. In all cases where a transglycosylation product was detected, a β-1,4-glycosidic linkage has been formed as determined by NMR spectroscopy of purified products. Glycosynthase reactions with selected acceptors were further studied to measure initial rates as summarized in Table 3. These reactions were carried out at low enzyme concentrations in order to monitor the initial formation of products, so yields after 24 h are only 40−50%. In most cases, after the formation of the initial transglycosylation product (labeled condensation in Table 3), a second compound was detected, which corresponded to an elongation product arising from a second coupling of the α-laminaribiosyl fluoride donor with the first condensation product (as demonstrated by NMR and MS). They are minor compounds detected only after 2−4 h of reaction time, which account for less than 5% of the total transglycosylation products at 24 h.

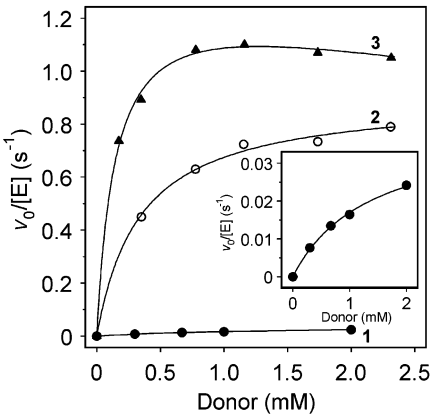
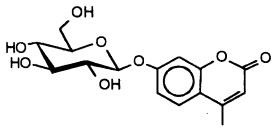
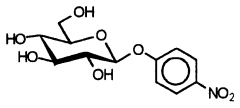
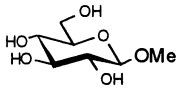
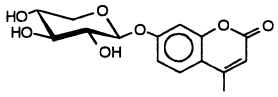
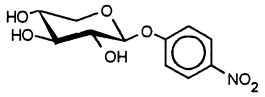
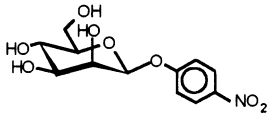
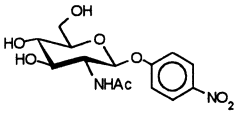
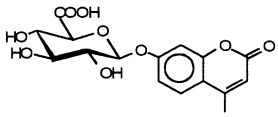
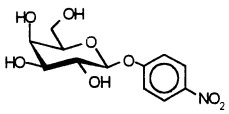
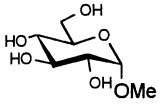


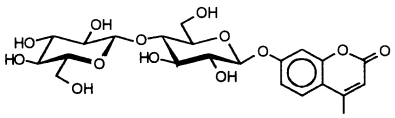
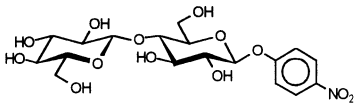
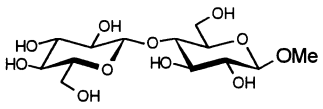
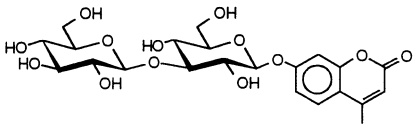
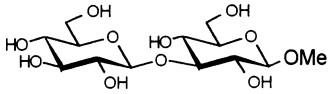
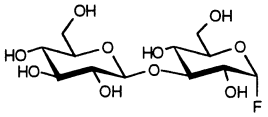
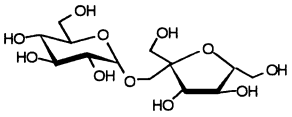
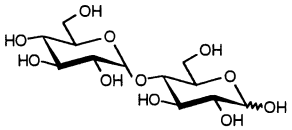
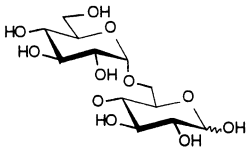
FIGURE 1: Kinetics of E134A glycosynthase-catalyzed condensations of donors **1** (●), **2** (○), and **3** (▲) and acceptor **14** in maleate buffer (pH 7.0) and 0.1 mM $CaCl_2$ at 35 °C, where [E134A] = 0.03−0.3 mM and [acceptor **14**] = 7.8 mM. The inset shows a magnification of kinetics with donor **1**. Kinetic parameters are given in Table 1.

Preparative glycosynthase reactions were carried out at a 1:5 donor:acceptor molar ratio and higher enzyme concentrations (10−30 μM) under the same experimental conditions used in the kinetic experiments. Product isolation followed two different protocols depending on the acceptor compound. For monosaccharide acceptors (**4** and **8**), the reaction mixture was directly purified by reverse-phase chromatography to afford the condensation products in 88 and 71% yields, respectively. For disaccharide acceptors (**14** and **16**−**18**), the reaction mixture was fully acetylated and purified by silica gel chromatography, and the per-O-acetylated products were treated with sodium methoxide in methanol to give the condensation products in 25−85% yield (Table 4). All compounds were characterized by MS and ^{1}H and ^{13}C NMR spectrometry. In all cases, a new β-1,4-glycosidic bond was formed. For gluco-oligomers (compounds **22** and **24**−**27**, Table 4), the ^{1}H NMR spectra show a new signal in the anomeric region that can be assigned to H-1 in β-1,4-linkages (δ 4.6−4.4 ppm, J = 7.8−8.1 Hz), whereas the signal corresponding to the carbon involved in the new glycosidic bond appears at δ 77−79 ppm in the ^{13}C NMR spectra, which is characteristic of a C4-O-linked glucoside. In contrast, the C3 signal in a C3-O-linked glucoside would appear at δ 86.5−87.1 ppm (*25, 29*). Likewise, in the condensation product with the xyloside **8** (compound **23**, Table 4), the ^{13}C NMR signal at δ 76.8 ppm corresponds to a C4-O-linked xyloside (a C3-O-linked xyloside would give a signal at δ 84 ppm for C3) (*37*−*39*).

Processes Competing with Donor−*Acceptor Condensation.* To evaluate the extent to which other processes may compete with the desired enzyme-catalyzed condensation, the model reaction between trisaccharide donor **2** and acceptor **15** (Scheme 4) was analyzed in detail. These processes were kinetically characterized by combining HPLC monitoring of product formation and fluoride anion release by a selective fluoride electrode.

Table 2: Glycosynthase Activity of the E134A 1,3-1,4-β-Glucanase Mutant[a]

	Acceptor (monosaccharides)	R
4		1.00
5		0.95
6		< 0.01
7		1.10
8		1.21
9		0.03
10		n.r.
11		n.r.
12		n.r.
13		n.r.

	Acceptor (disaccharides)	R
14		1.29
15		0.91
16		1.03
17		0.41
18		0.20
16		0.11
19		n.r.
20		n.r.
21		n.r.

[a] Relative yields of transglycosylation after reactions (R) for 24 h with the α-laminaribiosyl fluoride (**1**) donor and different acceptors. Conditions: 50 mM maleate buffer, pH 7.0, 0.1 mM $CaCl_2$, [donor] = 3 mM, [acceptor] = 6 mM, [E134A] = 3 μM, and 35 °C. n.r. means no reaction.

(a) Nonenzymatic Hydrolysis of the Donor. The α-glycosyl fluoride was incubated in maleate buffer under the same experimental conditions used in transglycosylation reactions (pH 7.0, 0.1 mM $CaCl_2$, and 35 °C) in the absence of enzyme and acceptor. The initial rates of spontaneous hydrolysis (measured by F^- release) at different α-glycosyl fluoride concentrations (0−15 mM) follow first-order kinetics with a rate constant k_H of 2.57×10^{-6} s^{-1}.

(b) Self-Condensation and Enzymatic Hydrolysis of the Donor. The α-glycosyl fluoride donor can be hydrolyzed by the enzyme, and also act as an acceptor (it contains a condensable free 4-OH group on the nonreducing end), leading to a dimer or self-condensation product. Both processes were analyzed by monitoring F^- release upon incubation of the donor with the enzyme in the absence of the acceptor. Rates are plotted against the donor concentration

Table 3: Initial Rates of Transglycosylation for the Reaction of α-Laminaribiosyl Fluoride (**1**) with Various Acceptors, Catalyzed by E134A 1,3-1,4-β-Glucanase[a]

			V_0/[E] (s^{-1})		yield (%) at 24 h	
acceptor	[D] (mM)	[A] (mM)	condensation	elongation	condensation	elongation
Glc-MU (**4**)	3	6	0.0341	0.0039	43	3
Glc-MU (**4**)	1	2	0.0061	nd[b]	33	–
Glc-pNP (**5**)	3	6	0.0324	0.0022	39	2
Xyl-MU (**7**)	1	2	0.0046	nd[b]	36	–
Xyl-pNP (**8**)	3	6	0.026	0.0019	52	5
Glcβ4Glc-MU (**14**)	3	6	0.0418	0.0042	51	4
Glcβ4Glc-pNP (**15**)	3	6	0.0122	0.0011	36	3
Glcβ3Glc-MU (**17**)	3	6	0.0084	0.0010	19	2

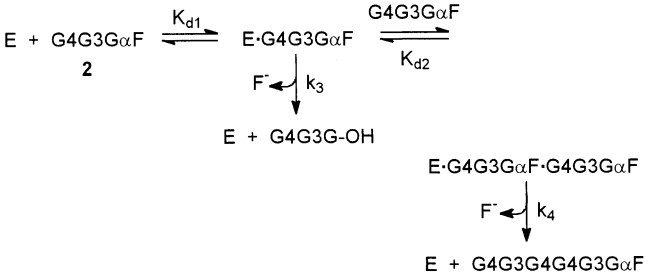
[a] Conditions: 50 mM maleate buffer, pH 7.0, 0.1 mM $CaCl_2$, [E134A] = 3 μM, and 35 °C. [b] Not detected.

Table 4: Preparative Yields for E134A 1,3-1,4-β-Glucanase-Catalyzed Glycosynthase Reactions of α-Laminaribioside Fluoride (**1**) with Different Mono- and Disaccharide Acceptors

		purification	NMR[c]		overall yield
acceptor[a]	condensation product	method[b]	^{1}H	^{13}C	(%)[d]
Glc-MU (**4**)	Glcβ3Glcβ4Glc-MU (**22**)	A	4.60 (8.1)	78.8	88
Xyl-pNP (**8**)	Glcβ3Glcβ4Xyl-pNP (**23**)	A	4.61 (8.1)	76.8	71
Glcβ4Glc-MU (**14**)	Glcβ3Glcβ4Glcβ4Glc-MU (**24**)	B	4.60 (8.7)	78.7	75
Glcβ4Glc-OMe (**16**)	Glcβ3Glcβ4Glcβ4Glc-OMe (**25**)	B	4.43 (7.8)	78.7	83
Glcβ3Glc-MU (**17**)	Glcβ3Glcβ4Glcβ3Glc-MU (**26**)	B	4.56 (7.8)	80.7	25
Glcβ3Glc-OMe (**18**)	Glcβ3Glcβ4Glcβ3Glc-OMe (**27**)	B	4.54 (7.8)	79.1	55

[a] Five equivalents of acceptor relative to donor **1**. [b] Purification method: (A) reverse-phase chromatography and (B) acetylation (Ac_2O/py), chromatography (SiO_2), and de-O-acetylation (MeONa/MeOH) as described in detail in Materials and Methods. [c] New NMR signal in the condensation product. ^{1}H is the H-1 signal in parts per million (J in hertz). ^{13}C is the C-4 signal in parts per million, for the newly formed β-1,4-glycosidic linkage. [d] Overall yield in isolated condensation product relative to donor substrate.

in Figure 2. They follow a saturation-like curve with the following apparent kinetic parameters: $K_M \approx 7.36$ mM and $k_{cat} \approx 2.73$ s^{-1}. Compared to this rate constant, the rate of nonenzymatic hydrolysis determined before is negligible. If one assumes that enzymatic hydrolysis and donor self-condensation take place simultaneously, the measured fluoride release rates arise from both competing processes. The simplest kinetic model that accounts for the observed saturation-like curve d[F^-]/dt as a function of donor concentration is



with a rate expression

$$\frac{d[F^-]}{dt} = E_0 \frac{k_4[S]^2 + k_3 K_{d2}[S]}{K_{d1}K_{d2} + K_{d2}[S] + [S]^2} \qquad (2)$$

where F^- is the total fluoride ion released and S is donor **2**.

Fitting the data to eq 2 (as shown in Figure 2) gives a hydrolysis constant (k_3) of 0.14 s^{-1}, a self-condensation constant (k_4) of 3.11 s^{-1}, a dissociation constant of the enzyme−donor complex (K_{d1}) of 0.11 mM, and a dissociation constant of the ternary complex (K_{d2}) of 10.58 mM. Accordingly, self-condensation is faster than enzymatic hydrolysis ($k_4 > k_3$), but the extent of both processes depends on donor concentration:

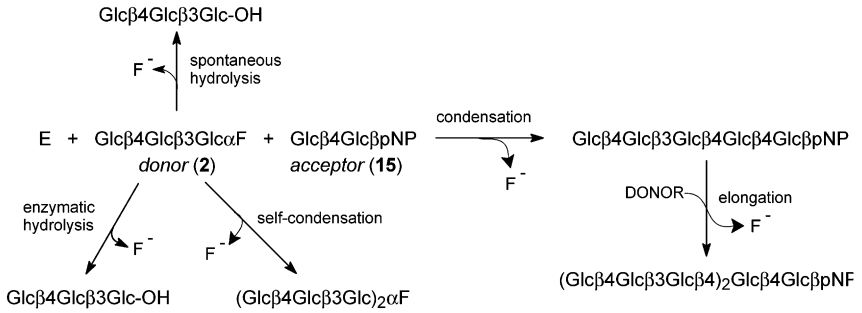
$$\frac{v_{SC}}{v_H} = \frac{k_4}{k_3}\frac{[S]}{K_{d2}} \qquad (3)$$

where v_{SC} is the rate of self-condensation and v_H is the rate of enzymatic hydrolysis. In this case, self-condensation becomes the main process at >0.48 mM S.

(c) Donor−Acceptor Condensation. At a constant donor (**2**) concentration (2 mM) and varying acceptor (**15**) concentrations, the rate of condensation product formation (determined by HPLC) follows saturation kinetics (Figure 3) with a k_{cat}^{app} of 1.7 s^{-1} and a K_M ^{app}(acceptor) of 11 mM. Taken together with the kinetic parameters for the donor (Table 1), the K_M^{app} of 11 mM for the acceptor is approximately its K_M value since this experiment was carried out at saturating donor concentrations [$>6K_M$(donor)]. As compared to the donor self-condensation parameters obtained above, k_{cat}^{app} is lower than k_4 and K_M^{app} is higher than K_{d2}. In terms of second-order rate constants, a k_{cat}^{app}/K_M^{app} of 0.15 mM^{-1} s^{-1} is half the k_4/K_{d2} value of 0.29 mM^{-1} s^{-1} for self-condensation, indicating that the trisaccharide **2** is a better acceptor than *p*-nitrophenyl β-cellobioside (**15**).

The total F^- release for the donor **2** + acceptor **15** reaction follows the curve shown in Figure 3; at 0 mM acceptor, the rate of F^- release corresponds to the rate of self-condensation and enzymatic hydrolysis of the donor ($v_0/[E]_0 = 0.61$ s^{-1}), whereas at high acceptor concentrations, it is approximately equal to the rate of formation of the condensation product by HPLC. It means that condensation becomes the main process with an excess of acceptor because it competes with the donor for the acceptor subsites of the enzyme, thus decreasing the rate of self-condensation of the donor.

Scheme 4: Competing Processes in the Glycosynthase Reaction of E134A 1,3-1,4-β-Glucanase with Donor **2** and Acceptor **15**[a]



[a] Reactions were monitored by fluoride ion release and formation of chromogenic products (pNP-glycosides; see the text).

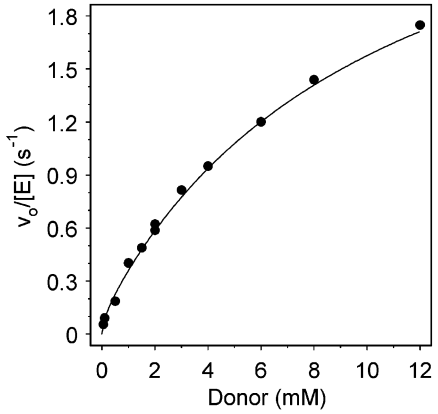


FIGURE 2: Kinetics of self-condensation and enzymatic hydrolysis of donor **2** catalyzed by E134A 1,3-1,4-β-glucanase in 50 mM maleate buffer (pH 7.0) and 0.1 mM $CaCl_2$ at 35 °C, where [enzyme] = 0.15 μM and [donor **2**] = 0.05−12 mM. $v/[E]_0$ is the rate of fluoride ion release.

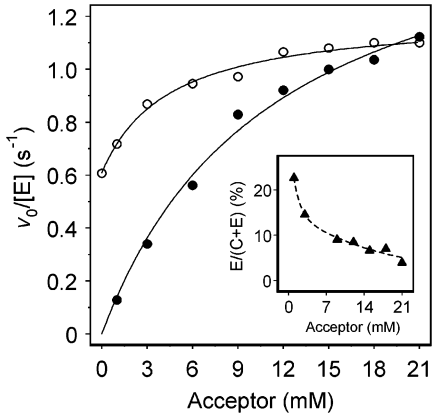


FIGURE 3: Kinetics of donor−acceptor condensation catalyzed by E134A 1,3-1,4-β-glucanase: (●) condensation product by HPLC and (○) total F^- released by the fluoride selective electrode. The inset shows the percentage of elongation product determined by HPLC. $E/(C + E)$, where E is the rate of elongation and C the rate of condensation. Conditions: 50 mM maleate buffer (pH 7.0), 0.1 mM $CaCl_2$, 35 °C, [enzyme] = 0.04−0.16 μM, [donor **2**] = 2 mM, and [acceptor **15**] = 0−21 mM.

(d) Elongation Products. The accumulated condensation product may react as an acceptor with the donor to yield elongation products (Scheme 4). For the same experiment where donor−acceptor condensation was analyzed above, monitoring of the elongation product by HPLC gives the rate dependence on acceptor concentration shown in the inset of Figure 3. This process depends on the donor:acceptor concentration ratio; at low acceptor concentrations, the elongation rate is up to 20% of those of the overall processes,

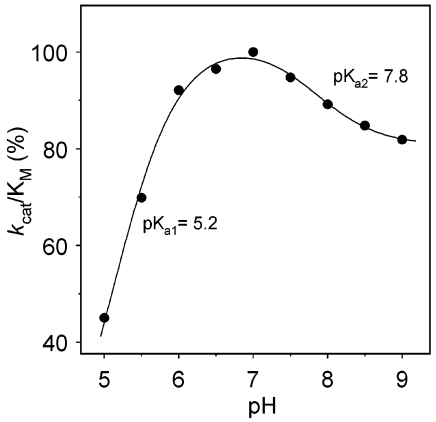
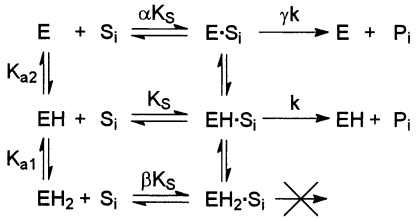


FIGURE 4: pH dependence of k_{cat}/K_M(donor) for the glycosynthase-catalyzed condensation of donor **1** and acceptor **15** by E134A 1,3-1,4-β-glucanase in 50 mM citrate−50 mM phosphate buffer and 0.1 mM $CaCl_2$, with a constant ionic strength (0.5 M) at 35 °C, where [enzyme] = 3 μM, [donor **1**] = 0.1−1 mM, and [acceptor **15**] = 24 mM. The kinetic pK_a values (5.2 and 7.8) were obtained by fitting the data to eq 4.

but it is only 2−4% at high acceptor concentrations. The elongation:condensation ratio for given donor and acceptor initial concentrations is kept constant during the reaction time (data not shown). No longer oligomers other than the first elongation product were detected.

pH Dependence of the Glycosynthase Activity. $(k_{cat}/K_M)_{donor}$ values for the E134A 1,3-1,4-β-glucanase-catalyzed condensation reaction of α-laminaribiosyl fluoride (**1**) with acceptor **15** were determined (by HPLC) over the pH range of 5.0−9.0. Reactions were carried out at a saturating acceptor concentration (24 mM) and different donor concentrations (0−1 mM) below its K_M value in citrate−phosphate buffer at a constant ionic strength. Under these conditions, k_{cat}/K_M values for the donor were obtained as the slope of $v_0 = (k_{cat}/K_M)_{donor}$[donor]. The plot of $(k_{cat}/K_M)_{donor}$ versus pH shown in Figure 4 is rather complex, corresponding to a double-ionization process. This behavior is described by the following kinetic scheme (*40*) in which two ionic forms of the enzyme are active:



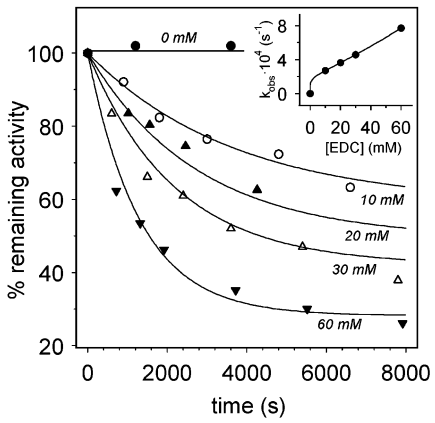


FIGURE 5: Kinetics of enzyme inactivation by EDC at pH 5.5 and 25 °C. Remaining activity (%) vs time of inactivation at different EDC concentrations (0−60 mM). Data were fitted to eq 7. The inset shows k_{obs} values at each EDC concentration (eq 8).

where E is the enzyme with three different ionization states (E, EH, and EH_2) related by two pK_a values (pK_{a1} and pK_{a2}), S_i are the substrates (donor and acceptor), and P_i is the transglycosylation product. At low pH, the enzyme has no activity (inactive EH_2 form); at pH ≈7, a maximum k_{cat}/K_M value is reached, whereas at high pH, an appreciable residual activity is maintained, indicating that both ionization states EH and E are active with regard to glycosynthase activity, EH being more active than E. The equation describing this model is

$$(k_{cat}/K_M)_{donor} = A\frac{(1 + B \times 10^{pH-pK_{a2}})}{1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}}} \quad (4)$$

where $A = k/K_S$ and $B = \gamma/\alpha$. The calculated kinetic pK_a values obtained by nonlinear regression fitting of the data to eq 4 are 5.2 ± 0.1 and 7.8 ± 0.1.

Enzyme Inactivation by EDC. The participation of carboxyl groups in the mechanism of the E134A glycosynthase was investigated by analyzing the kinetics and pH dependence of enzyme inactivation by a water-soluble carbodiimide (EDC) (*41*, *42*). E134A 1,3-1,4-β-glucanase was incubated with different concentrations of EDC (0−120 mM) at pH 5.5 and 25 °C, and the reaction was quenched at different time intervals to measure the residual glycosynthase activity. This treatment resulted in a time-dependent enzyme inactivation (Figure 5) up to 23% residual activity with 120 mM EDC after reaction for 1 h. The inactivation process proved to be irreversible since no change in residual activity was obtained after extensive dialysis. Under the same experimental conditions, inactivation by EDC in the presence of glycine methyl ester (1.2 M) as a nucleophile gave a residual activity of 8%. A plot of the remaining glycosynthase activity as a function of time of inactivation at different EDC concentrations (Figure 5) obeys an exponential equation:

$$\text{act}(\%) = Ae^{-k_{obs}t} + B \quad (5)$$

where A is the amplitude, k_{obs} the inactivation constant, and B the residual activity after a long incubation time. Since no complete inactivation ($B > 0$) is obtained, the standard Levy's method for calculating the order of inactivation [as the slope of log(k_{obs}) vs log[I] for a single inactivation process $E + nI \rightarrow EI_n$, where I is the inactivator] (*43*) cannot be applied (*44*). These results suggest that modification of the glycosynthase enzyme occurs at least at two different types of carboxyl groups, one leading to a completely inactive enzyme but the other resulting in a partially active enzyme, and modification of either prevents the reaction on the other:

$$E + n_1EDC \xrightarrow{k_1} E^*$$

$$E + n_2EDC \xrightarrow{k_2} E1^*$$

where E* is completely inactive and E1* is partially active.

The observed remaining activity at any reaction time is given by

$$\text{act}(t) = \frac{a_E[E] + a_{E1^*}[E1^*]}{a_E[E]_0} \quad (6)$$

where a_E is the specific activity of the unmodified enzyme and a_{E1^*} the specific activity of the modified but partially active enzyme. According to this reaction scheme, the remaining activity as a function of time is expressed as

$$\text{act}(t) = \left(1 - \frac{a_{E1^*}}{a_E}\frac{k'_2}{k'_1 + k'_2}\right)e^{-(k'_1+k'_2)t} + \frac{a_{E1^*}}{a_E}\frac{k'_2}{k'_1 + k'_2} \quad (7)$$

where $k'_1 = k_1[EDC]^{n_1}$ and $k'_2 = k_2[EDC]^{n_2}$, and the constants in eq 5 become

$$k_{obs} = k'_1 + k'_2 = k_1[EDC]^{n_1} + k_2[EDC]^{n_2} \quad (8)$$

$$A = 1 - \frac{a_{E1^*}}{a_E}\frac{k'_2}{k'_1 + k'_2} \quad (9)$$

$$B = \frac{a_{E1^*}}{a_E}\frac{k'_2}{k'_1 + k'_2} \quad (10)$$

The activity versus time data were fitted to eq 7 by nonlinear regression to obtain the fittings plotted in Figure 5 with the following numerical values for the constants: $k_1 = 1.87 \times 10^{-6}$, $k_2 = 1.53 \times 10^{-4}$, $n_1 = 1.34$, $n_2 = 0.18$, and $a_{E1^*}/a_E = 0.69$. k_{obs} (s^{-1}) (eq 8) can be derived from these as a function of EDC concentration as shown in the inset of Figure 5.

Even though carbodiimides react preferentially with carboxylic acid groups, they are also able to modify cysteine and tyrosine residues at slightly acidic pH (*45*, *46*). Modification of cysteines is discarded since 1,3-1,4-β-glucanase has two Cys residues involved in a disulfide bond. Tyrosine residues modified by EDC yield an *O*-arylisourea derivative, which incubation with an excess of hydroxylamine can revert (*46*). Treatment of an EDC-modified enzyme (after extensive dialysis to remove an excess of reagents) with 0.5 M hydroxylamine did not recover glycosynthase activity. Moreover, nitration of tyrosines in the free enzyme with tetranitromethane (TNM) at pH 8 for 10 h indicated that all 17 Tyr residues of the protein were modified [monitored spectrophotometrically at 428 nm (λ)], but only 15% reduction in glycosynthase activity was measured for the TNM-treated enzyme (data not shown). Therefore, modification of Tyr residues does not have a major effect on glycosynthase activity, so the observed inactivation by EDC

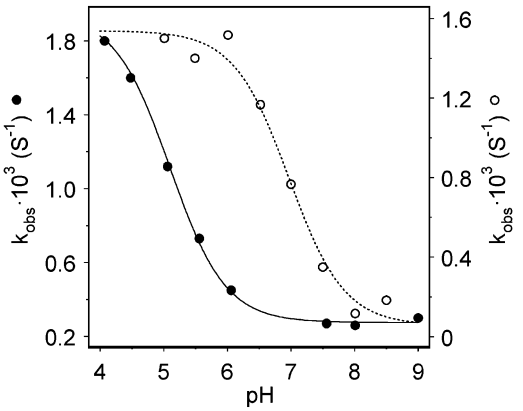


FIGURE 6: pH dependence of glycosynthase inactivation by EDC. k_{obs} vs pH data were fitted to eq 11 to give a pK_a value of 5.3 ± 0.2: (●) E134A glycosynthase and (○) wild-type enzyme [inactivation of hydrolase activity (*33*)]. Conditions: ternary buffer at a constant ionic strength (pH 4−9), [E134A] = 26 μM, and [EDC] = 120 mM.

can be ascribed to modification of carboxylic acid residues. In addition, EDC inactivation is due to active site residues since inactivation was partially prevented by the hexasaccharide Galβ4Glcβ4Glcβ3Glcβ4Glcβ4GlcβOMe (*24*), a specific ligand of 1,3-1,4-β-glucanases. Treatment of the glycosynthase with EDC (120 mM) at pH 5.0 in the presence of the ligand (0−12 mM) protected the enzyme from inactivation in a concentration-dependent manner. At 12 mM ligand, the level of inactivation after reaction for 1.5 h was half of that obtained in its absence.

The pH dependence of inactivation by EDC (k_{obs} vs pH) is shown in Figure 6. It follows a single titration curve with fast inactivation rate constants at acidic pH values. Data were fitted to eq 11, which corresponds to a single ionization process plus an independent term (*b*) to account for the residual k_{obs} at high pH values

$$k_{obs} = \frac{(k_{obs})_{lim}}{1 + 10^{pH-pK_a}} + b \qquad (11)$$

where fitted parameters $b = 2.8 \times 10^{-4}$ s^{-1} and $(k_{obs})_{lim} = 1.7 \times 10^{-3}$ s^{-1} and with an apparent pK_a value of approximately 5.3 ± 0.2.

E134A/E138A Double Mutant. Chemical Rescue of Hydrolase and Glycosynthase Activities. The residue acting as the general acid−base catalyst in the wild-type hydrolase reaction (Glu138 in *B. licheniformis* 1,3-1,4-β-glucanase) is proposed to function as the catalytic base in the glycosynthase mechanism (Scheme 2). This residue was mutated to Ala on the E134A glycosynthase to produce the double mutant E134A/E138A. Kinetics of the enzyme-catalyzed hydrolysis of the activated 2,4-dinitrophenyl glycoside substrate **29** are summarized in Table 5 and compared with those of the single alanine mutants (*21*). The double mutant behaves much like E134A (nucleophile mutant) with a 10^6-fold reduction in k_{cat} relative to that of the wild-type enzyme. The activity was restored when applying a chemical rescue methodology as previously shown on E134A and E138A single mutants. Incubation of the activated substrate 2,4-dinitrophenyl glycoside **29** with E134A/E138A in the presence of 2 M sodium azide resulted in a fast aglycon release (UV detection). The reaction was monitored by ^{1}H NMR

Table 5: Hydrolase Activity of Wild-Type and Active Site Mutants of 1,3-1,4-β-Glucanase with the Substrate 2,4-Dinitrophenyl 3-*O*-β-Cellobiosyl-β-D-glucoside (**29**)[a]

enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
wild-type	603 ± 8	0.20 ± 0.01	(3.0 ± 0.2) × 10^6
E134A	(7.1 ± 0.1) × 10^{-4}	0.16 ± 0.02	4.4 ± 0.5
E138A	0.256 ± 0.005	0.22 ± 0.02	(1.2 ± 0.1) × 10^3
E134A/E138A	(7.7 ± 0.2) × 10^{-4}	0.3 ± 0.1	2.6 ± 0.8

[a] Conditions: 6.5 mM citrate/87 mM phosphate buffer, pH 7.2, 0.1 mM $CaCl_2$, 30 °C, [Glcβ4Glcβ3Glc-2,4DNP (**29**)] = 0.01−2 mM, enzyme concentrations of 0.1 nM to 0.14 μM (wild type), 5 nM to 1 μM (E134A), 10 nM to 1 μM (E138A), and 5 μM (E134A/E138A). Data for the wild type and single mutants E134A and E138A were reported in ref *21*.

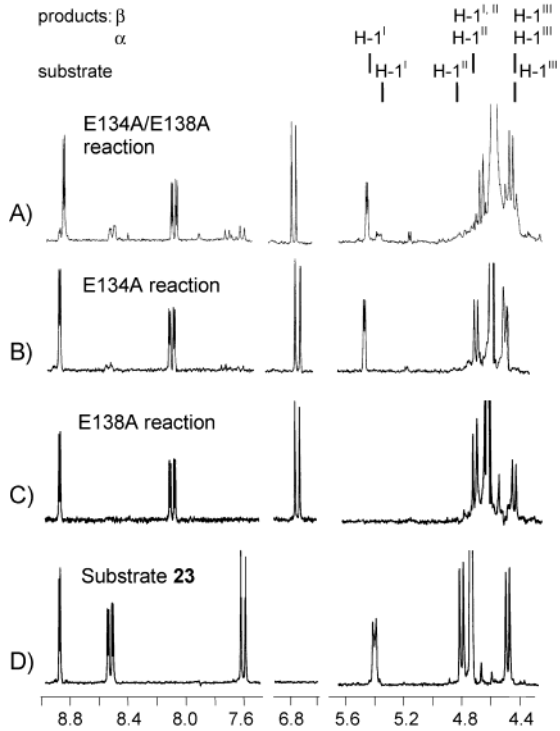


FIGURE 7: ^{1}H NMR spectra of the reactions of substrate Glcβ4Glcβ3Glc-2,4DNP (**29**) with 1,3-1,4-β-glucanase E134A/E138A (A), E134A (B), and E138A (C) mutants in the presence of sodium azide, in deuterated phosphate buffer at 30 °C and pD 7.2 for 72 h. [E] = 34 μM, [substrate] = 11.8 mM, and [azide] = 2 M. δ and *J* values: 5.53 ppm and 3.6 Hz for H-1^{I} α-glycosyl azide, 5.45 ppm and 7.2 Hz for H-1^{I} substrate, 4.81 ppm and 8.1 Hz for H-1^{II} substrate, 4.76 ppm and 8.1 Hz for H-1^{I,II} β-glycosyl azide, 4.70 ppm and 8.1 Hz for H-1^{II} α-glycosyl azide, 4.49 ppm and 8.0 Hz for H-1^{III} α-glycosyl azide, and 4.48 ppm and 8.1 Hz for H-1^{III} substrate and β-glycosyl azide, respectively. Aromatic signals at δ 8.90 (2.7 Hz) for H-3′ substrate and H-3′ 2,4-dinitrophenol, δ 8.55 (2.7 and 9.3 Hz) for H-5′ substrate, δ 8.10 (3.0 and 9.6 Hz) for H-5′ 2,4-dinitrophenol, δ 7.62 (9.6 Hz) for H-6′ substrate, and δ 6.72 (9.6 Hz) for H-6′ 2,4-dinitrophenol.

spectroscopy (in D_2O at pD 7.2 and 25 °C) to follow the time progress and for structure determination of the final products. Results are shown in Figure 7, together with control reactions that included the single E134A mutant (which yielded the α-glycosyl azide product, Figure 7B) and the single E138A mutant (which gave the β-glycosyl azide, Figure 7C) (*21*). A doublet at δ 5.53 ppm (J = 3.6 Hz) develops at the same rate at which the doublet corresponding to H-1^{I} of the substrate (δ 5.45 ppm, J = 7.2 Hz) disappears. Little hydrolysis product (H-1^{I} signal at δ 5.23 ppm and *J*

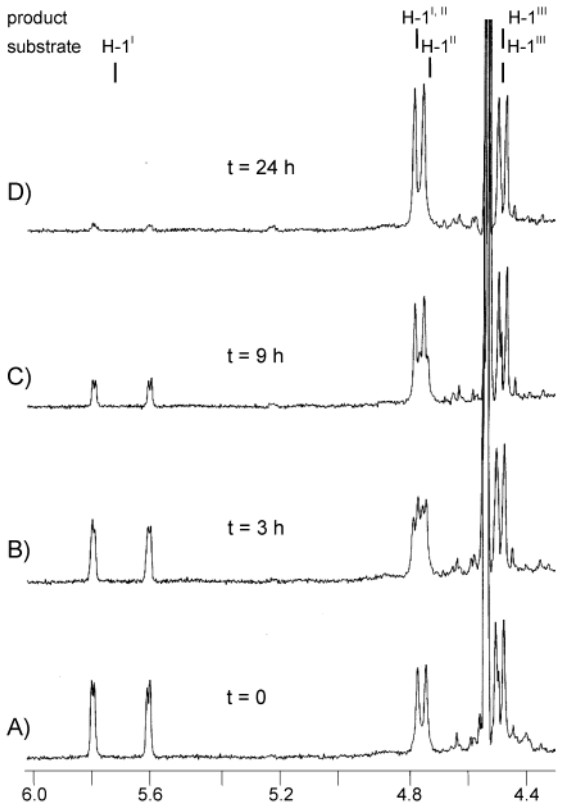


FIGURE 8: ^{1}H NMR monitoring of the reaction of Glcβ4Glcβ3Glc-αF (**2**) with the 1,3-1,4-β-glucanase E134A/E138A mutant in the presence of sodium azide, where 34 μM E, 15 mM substrate, and 2 M azide were incubated in deuterated phosphate buffer at 35 °C and pD 7.4: Glcβ4Glcβ3Glc-αF substrate at time zero (A) and after 3, 9, and 24 h (B−D, respectively). δ and J values: 5.70 ppm and 53.4 Hz for H-1^{I} substrate, 4.75 ppm and 8.1 Hz for H-1^{I,II} β-glycosyl azide, and 4.49 ppm and 7.8 Hz for H-1^{III} substrate and β-glycosyl azide, respectively.

= 3.5 Hz for the α-anomer and δ 4.66 ppm and J = 7.5 Hz for the β-anomer) is detected. By comparison with the adduct produced in the E134A reaction (Figure 7B), the main product of chemical rescue of the double mutant is the α-glycosyl azide, but its rate of formation is half of that with the single E134A mutant.

Once proper folding and the ability to bind substrate have been proven for the E134A/E138A mutant, it was tested as a glycosynthase. Addition of the double mutant to a mixture of α-laminaribiosyl fluoride (**1**) and acceptor **14** under typical glycosynthase conditions yielded no transglycosylation product (HPLC and TLC monitoring). It agrees with the proposed role of Glu138 in the E134A glycosynthase as a general base by activating the hydroxyl group of the acceptor.

The reaction with azide as the nucleophile acceptor not requiring general base catalysis was analyzed. In a preliminary experiment, incubation of the α-laminaribiosyl fluoride donor (**1**) with the E134A/E138A mutant and 2 M sodium azide produced β-laminaribiosyl azide in quantitative yield (^{1}H NMR monitoring, δ 4.75 ppm and J = 9.3 Hz for H-1^{I} and δ 4.72 ppm and J = 8.7 Hz for H-1^{II} of the product). However, it was not conclusive since the same adduct was also formed at approximately the same rate in the absence of enzyme because of the high chemical reactivity of the fluoride. Using a better glycosyl donor, the trisaccharyl fluoride **3**, the enzyme-catalyzed nucleophilic reaction with azide was much faster. ^{1}H NMR monitoring (Figure 8)

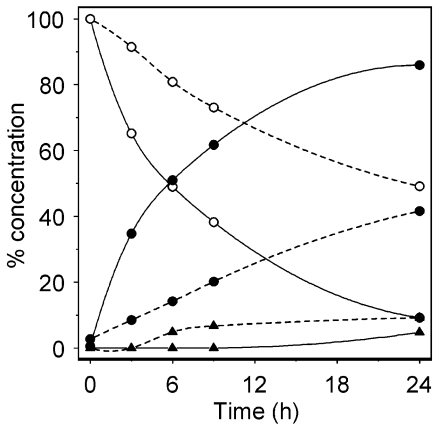


FIGURE 9: Time course analysis of the reactions of Glcβ4Glcβ3Glc-αF (**2**) substrate and azide in the presence (—) and absence (- - -) of the 1,3-1,4-β-glucanase E134A/E138A mutant by ^{1}H NMR: (○) substrate, (●) β-glycosyl azide, and (▲) hydrolysis products. Conditions: [substrate] = 15 mM, [azide] = 2 M, with or without 34 μM E, 50 mM deuterated phosphate buffer, pD 7.4, and 35 °C.

showed a doublet at δ 4.75 ppm (J = 8.1 Hz), corresponding to H-1^{I} of the β-glycosyl azide product, that developed at the same rate at which the H-1^{I} and H-1^{II} signals of the donor substrate at δ 5.70 ppm (J = 53.4 Hz) and δ 4.74 ppm (J = 7.5 Hz), respectively, disappeared. The time course of the ^{1}H NMR integrals is presented in Figure 9. After 24 h, the β-azide product was obtained in 90% yield, whereas the chemical reaction (in the absence of enzyme) yielded only 40% of the same product under identical experimental conditions.

Attempts to recover glycosynthase activity of the double mutant by adding exogenous bases failed. Different bases such as ammonia, methylamine, cyanomethylamine, glycine methyl ester, and sodium formate at 1 M were added to a mixture of the α-laminaribiosyl fluoride (**1**) donor and acceptor **4** at different pH values (7−9.2) and 35 °C with the E134A/E138A mutant. No condensation product was detected in any case by HPLC.

DISCUSSION

Glycosynthase Activity and Specificity. *B. licheniformis* 1,3-1,4-β-glucanase was the first *endo*-glycosidase to be engineered as a glycosynthase (E134A mutant), catalyzing the condensation of α-laminaribiosyl fluoride with 4-methylumbelliferyl β-glucoside and β-cellobioside in 80−90% yields (*11*). Specificity and kinetic studies are analyzed here to explore further synthetic applications and better understand the mechanism of glycosynthases.

Donor and acceptor specificities are summarized in Tables 1−3. α-Glucosyl fluoride is not a donor, and the enzyme requires a disaccharide occupying subsites −1 and −2 for detectable glycosynthase activity. For a series of donors with an increasing degree of polymerization ([Glcβ4]$_n$Glcβ3GlcαF, n = 0−2), k_{cat}/K_M values (Table 1) increase from disaccharide **1** to tetrasaccharide **3**, because of increasing k_{cat} and decreasing K_M values, as expected for an *endo* enzyme and in agreement with subsite mapping analysis of the wild-type enzyme (*33*). Different mono- and disaccharide derivatives were assayed to define the acceptor specificity (Tables 2 and 3). Methyl β-glucoside **6** is a poor acceptor, whereas 4-methylumbelliferyl and 4-nitrophenyl β-glucosides (**4** and **5**, respectively)

react readily. It indicates that subsite +1 has a low affinity for productive binding to a monosaccharide; the acceptor must occupy subsite +2 with an aromatic aglycon or with another glucosyl unit as seen with disaccharide acceptors **14**−**18**. The 5-hydroxymethyl group is not required since aryl β-xylobiosides **7** and **8** are also good acceptors with initial condensation rates similar to those of the homologous glucosides (Table 3). A 5-carboxyl group is not accepted (compound **11**) as well as other configurations at C2 (*manno*, **9**) or C4 (*galacto*, **12**). α-Glucosides (**13** and **19**−**21**) do not react at all, indicating that α-linked saccharides do not bind. 2-Acetamido-2-deoxy-β-glucoside (**10**) was not glycosylated, in agreement with modeling studies which predict a hydrogen bond interaction between the glucosyl 2-OH group and a glutamate residue of the protein without room to accommodate a larger substituent (*47*).

On disaccharides, the cellobiosides **14**−**16** are better acceptors than the laminaribiosides **17** and **18**, probably as a consequence of a competition of laminaribioside acceptors with the α-donor for the same subsites −2 and −1, rather than as a consequence of the higher affinity of the cellobiosides for subsites +1 and +2. Subsite mapping analysis on the wild-type enzyme has shown that subsites +1 and +2 can accommodate both laminaribiosyl and cellobiosyl units with similar affinities (*19*, *48*). When comparing the methyl and 4-methylumbelliferyl glycosides of the same series, **14** versus **16** and **17** versus **18**, we find that the disaccharides containing an aromatic aglycon are slightly better acceptors than the corresponding methyl glycosides, indicating that some additional interaction occurs at the edge of the binding site cleft after subsite +2.

Preparative reactions with α-laminaribiosyl fluoride (**1**) and the acceptors shown in Table 4 yielded condensation products where a new β-1,4-glycosidic bond is formed, following the same specificity as the wild-type hydrolase activity. It also illustrates the high regioselectivity of *endo*-glycosynthases [shown for this enzyme and for E197 Cel7B cellulase from *H. insolens* (*16*)] as compared to the *exo*-acting glycosynthases reported so far where mixtures of regioisomers are often obtained (*10*, *12*, *13*). Condensation yields depend on the acceptor compound (Table 4); at a 1:5 donor:acceptor ratio, aryl monosaccharides and cellobiosides give 70−90% yields, whereas laminaribiosides result in lower yields (25−55%) due to substrate inhibition as a consequence of competition for the donor subsites of the enzyme.

Competing Reactions: Transglycosylation versus Self-Condensation. As observed with most of the current glycosynthases, the donor−acceptor condensation reaction is often accompanied by other side reactions. The glycosyl fluoride donor and the transglycosylation product may also act as acceptors provided that the configuration of the condensable hydroxyl group on the nonreducing end of the donor has the same stereochemistry as the normal acceptor, leading to self-condensation of the donor or elongation of the transglycosylation product, respectively. The extent of each process is analyzed by kinetic measurements of the model reaction between trisaccharyl fluoride donor **2** and cellobioside acceptor **15** (Scheme 4). In the absence of the acceptor, the rate constant of self-condensation is higher than the rate constant of enzymatic hydrolysis of the donor. For the overall system when the donor, acceptor, and enzyme are being mixed, the competition between donor self-condensation and donor−acceptor condensation depends on their relative affinities and concentrations. Because the trisaccharyl donor **2** is a better acceptor than the cellobioside **15** in this case, donor self-condensation is an important process at low acceptor concentrations; however, it becomes negligible with an excess of acceptor, and the condensation product reaches a 70−80% yield at a 1:5 donor:acceptor molar ratio. Likewise, further elongation of the condensation products (by a second coupling with the donor) may account for up to 20% of the overall processes at low acceptor concentrations, but the level of elongation is reduced to 2−4% with an excess of acceptor (1:5 donor:acceptor molar ratio) (Figure 3). It is therefore important to modulate these different reactions for an efficient use of the glycosynthase methodology in preparative synthesis of target oligosaccharides.

Besides the use of an excess of acceptor to minimize self-condensation and elongation, other strategies, which are highly dependent on the relative binding affinities of the donor and acceptor for the same acceptor site, have been devised and tested previously with the E134A glycosynthase, and also successfully used with another *endo*-glycosynthase, the E197A mutant of cellulase Cel7B from *H. insulens* (*16*, *49*).

(a) Selection of a Donor with a Different Configuration of the Hydroxyl Group that Normally Acts as an Acceptor (i.e., a galactosyl instead of a glucosyl residue on the nonreducing end of the donor). This strategy depends on how strict the specificity of the enzyme is at the donor subsites. Subsite −3 of *B. licheniformis* 1,3-1,4-β-glucanase accepts a galactosyl residue with almost the same affinity as a glucosyl residue, and the corresponding trisaccharyl fluoride Galβ4Glcβ3GlcαF as a donor in the E134 glycosynthase reaction gave a single condensation product with mono- and disaccharide acceptors in high yields (*50*).

(b) Use of a Temporary Protecting Group on the "Acceptor" Hydroxyl Group of the Donor. As shown for the E197A Cel7B glycosynthase, a 4″-tetrahydropyranyl (THP)-protected α-cellobiosyl fluoride is a good donor that is unable to self-condense (*49*). The same protecting group was applied to the E134A glycosynthase reaction in which the corresponding tetrasaccharide donor protected at the 4-OH group on the nonreducing end gave a single condensation product with the methyl β-cellobioside acceptor in 85% yield (*24*). After the glycosynthase-catalyzed condensation, the THP protecting group is removed by acid treatment.

Mechanism. It is commonly assumed that the mechanism of the glycosynthase reaction proceeds by general base catalysis in which the same residue that acts as a general acid in the hydrolase mechanism of the wild-type enzyme now plays the role of general base catalyst by increasing the nucleophilicity of the glycosyl acceptor (Scheme 2). We provide experimental proof of this mechanism by analyzing the pH dependence of glycosynthase activity, the kinetics of enzyme inactivation by a carboxyl-specific reagent (EDC), and the properties of the double mutant E134A/E138A (where Glu138 is the general acid−base in the wild-type hydrolase reaction).

The pH dependence of k_{cat}/K_M(donor) for the glycosynthase reaction of E134A is presented in Figure 4. At acidic pH (<5), the enzyme is inactive and activity increases at higher pH values up to a maximum at pH 7.0. It corresponds to

general base catalysis with a kinetic pK_a of 5.2. According to the proposed mechanism in Scheme 2, the carboxylate of Glu138 is the candidate to act as a base, and the pK_a of 5.2 will reflect its ionization. In the wild-type hydrolase mechanism, Glu138 has a pK_a of 7.0 as a general acid (*33*). The drop of 1.8 pH units in the E134A mutant profile and the shape of the pH profile corresponding to basic catalysis are attributed to the lack of the negatively charged nucleophile in the mutant. As already argued for the pK_a cycling of the general acid−base catalyst in retaining glycosidases, the residue that acts as general acid in the glycosylation step then becomes the general base in the deglycosylation step because of a decrease in pK_a when the covalent glycosyl−enzyme intermediate is formed (*51*). Charge distribution in the active site of the glycosynthase (nucleophile-less) mutant would be equivalent to that in the covalent glycosyl−enzyme intermediate for the wild-type reaction, enabling Glu138 to be ionized to act as a general base. At higher pH values, a pH-independent glycosynthase activity of the E134A mutant would be expected (a plateau in the k_{cat}/K_M vs pH profile) since the general base remains in its active ionization state. However, a decrease in activity following an ionization with a pK_a of 7.8 to a residual activity of 70% is observed (Figure 4). It indicates that the ionization of another residue, although not essential, affects the catalytic efficiency. We tentatively propose it to be Asp136, the third residue of the catalytic triad in the wild-type enzyme. Asp136 may be close to Glu138 to stabilize the ionized carboxylate to behave as a general base, as proposed in the deglycosylation step of the wild-type hydrolytic reaction. Ionization of Asp136 at high pH may render a less than optimal ionization/charge distribution of the active site that is reflected in a lower, but still important, glycosynthase efficiency.

Chemical modification of E134A glycosynthase by EDC supports the involvement of Glu138 as a general base. Glycosynthase activity is efficiently reduced by the water-soluble carbodiimide EDC (Figure 5). As other side reactions of carbodiimides with tyrosine and cysteine residues could be discarded, enzyme inactivation can be ascribed to modification of active site carboxyl groups. The process is irreversible and obeys a monoexponential decay with residual activity after a long incubation time. It contrasts with the EDC inactivation of the hydrolytic activity of the wild-type enzyme, where complete inactivation (no residual activity) was obtained, with first-order kinetics, an order of inactivation of 0.97, and a pH dependence of the inactivation with a pK_a of 7.0, assigned to the general acid Glu138 (*33*). This different behavior between hydrolase (wt) and glycosynthase (E134A) enzymes indicates a different accessibility and/or reactivity of the essential carboxylic acid residue in both enzymes, which can be explained as follows. The EDC reaction is initiated by protonation of the carbodiimide nitrogen by a carboxylic acid followed by nucleophilic attack on the central carbon atom by a carboxylate anion (*41*, *42*, *52*, *53*). Asp136 and Glu138 form a hydrogen bond in the glycosynthase enzyme. This hydrogen can be the proton donor, and one of both carboxylates might serve as the attacking nucleophile. Modification of Glu138 would yield a fully inactive enzyme. Modification of Asp136 would affect glycosynthase activity, but not inactivate the enzyme completely. Its derivatization would prevent modification of the neighboring Glu138, thus explaining the observed residual activity. Instead, in the wild-type enzyme, Glu138 and Asp136 are further apart from each other [Asp136 is H-bonding with the nucleophile Glu134 in the free wild-type enzyme (*22*)], and Glu138 is accessible to the reagent independent of modification of other active site carboxyl groups, thus leading to complete enzyme inactivation. Also consistent with inactivation due to modification of active site residues is the fact that the hexasaccharide Galβ4Glcβ4Glcβ3Glcβ4Glc4βGlcβOMe, a specific ligand of 1,3-1,4-β-glucanases (*24*), protects the glycosynthase from inactivation by EDC.

The pH profile of inactivation (k_{obs} vs pH, Figure 6) follows a titration curve with a pK_a of 5.3 ± 0.2 for the glycosynthase enzyme, when it was 7.0 ± 0.1 for the wild-type enzyme. Likewise, the kinetic pK_a (on k_{cat}/K_M) of the glycosynthase activity is 5.2 ± 0.1 (general base catalysis), whereas the kinetic pK_a (on k_{cat}/K_M) of the hydrolase activity of the wild type is 7.0 ± 0.1 (general acid catalysis). Both experiments are consistent in showing that the pK_a of Glu138 is 7.0 in the wild-type enzyme when it acts as a general acid in the hydrolase mechanism [glycosylation step (*33*)] and that the pK_a of this residue drops 1.7−1.8 pH units when the nucleophile is removed in the E134A mutant, after which it is able to behave as a general base in the glycosynthase enzyme.

Finally, to assign Glu138 as the actual general base, the double mutant E134A/E138A was analyzed. It is inactive as a hydrolase with a residual k_{cat} (substrate **29**) on the same order of magnitude as that obtained for the E134A mutant. Likewise, activity was rescued upon addition of sodium azide as an exogenous nucleophile, the product being the α-glycosyl azide as in the case of chemical rescue of the single E134A mutant. It demonstrates that the active site topology of the double mutant is maintained, and that cleavage of the glycosidic bond of the activated 2,4-dinitrophenyl glycoside **29** does not require protonic assistance by a general acid in the enzyme. The E134A/E138A mutant has no detectable glycosynthase activity. Addition of exogenous bases such as formate, methylamine, cyanomethylamine, glycine methyl ester, or ammonia did not rescue glycosynthase activity. However, sodium azide added as a stronger nucleophilic acceptor that does not require general base catalysis leads to the β-glycosyl azide adduct from the α-glycosyl fluoride donor (Figures 8 and 9).

Together, these results provide solid evidence of general base catalysis in the glycosynthase mechanism, with Glu138 as the catalytic residue directly participating as the base to increase the nucleophilicity of the saccharide acceptor for condensation with the donor as shown in Scheme 2.

SUPPORTING INFORMATION AVAILABLE

Spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Wong, C. H., and Whitesides, G. M. (1994) *Enzymes in synthetic organic chemistry, Tetrahedron Organic Chemistry Series*, Vol. 12, Pergamon Press, Oxford, U.K.
2. Koellr, K. M., and Wong, C. H. (2000) Emerging themes in medicinal glycoscience, *Nat. Biotechnol. 18*, 835−841.
3. Vocadlo, D. J., and Withers, S. G. (2000) Glycosidase-catalysed oligosaccharide synthesis, in *Carbohydrates in Chemistry and*

Biology (Ernst, B., Hart, G. W., and Sinaÿ, P., Eds.) pp 723−844, Wiley-VCH Verlag GmbH, Weinheim, Germany.
4. Koshland, D. E., Jr. (1953) Stereochemistry and the mechanism of enzymatic reaction, *Biol. Rev. 28*, 416−436.
5. Sinnott, M. L. (1990) Catalytic mechanisms of enzymic glycosyl transfer, *Chem. Rev. 90*, 1171−1202.
6. Davies, G., Sinnott, M. L., and Withers, S. G. (1998) Glycosyl transfer, in *Comprehensive Biological Catalysis* (Sinnott, M. L., Ed.) pp 119−209, Academic Press, San Diego.
7. Withers, S. G. (2001) Mechanism of glycosyl transferases and hydrolases, *Carbohydr. Polym. 44*, 325−337.
8. Williams, S. J., and Withers, S. G. (2002) Glycosynthases: Mutant glycosidases for glycoside synthesis, *Aust. J. Chem. 55*, 3−12.
9. Planas, A., and Faijes, M. (2002) Glycosidases and glycosynthases in enzymatic synthesis of oligosaccharides. An overview, *Afinidad 59*, 295−313.
10. Mackenzie, L. F., Wang, Q., Warren, R. A. J., and Withers, S. G. (1998) Glycosynthases: Mutant glycosidases for oligosaccharide synthesis, *J. Am. Chem. Soc. 120*, 5583−5584.
11. Malet, C., and Planas, A. (1998) From β-glucanase to β-glucansynthase: glycosyl transfer to α-glycosyl fluorides catalyzed by a mutant endoglucanase lacking its catalytic nucleophile, *FEBS Lett. 440*, 208−212.
12. Trincone, A., Perugino, G., Rossi, M., and Moracci, M. (2000) A novel thermophilic glycosynthase that effects branching glycosylation, *Bioorg. Med. Chem. Lett. 10*, 365−368.
13. Nashiru, O., Zechel, D. L., Stoll, D., Mohammadzadeh, T., Warren, A. J., and Withers, S. G. (2001) β-Mannosynthase: synthesis of β-mannosides with a mutant β-mannosidase, *Angew. Chem., Int. Ed. 40*, 417−420.
14. Jakeman, D. L., and Withers, S. G. (2002) On expanding the repertoire of glycosynthases: mutant β-galactosidases forming β-(1,6)-linkages, *Can. J. Chem. 80*, 866−870.
15. Okuyama, M., Mori, H., Watanabe, K., Kimura, A., and Chiba, S. (2002) α-Glucosidase mutant catalyzes "α-glycosynthase"-type reaction, *Biosci., Biotechnol., Biochem. 66*, 928−933.
16. Fort, S., Boyer, V., Grefee, L., Davies, G. J., Moroz, O., Christiansen, L., Schülein, M., Cottaz, S., and Driguez, H. (2000) Highly efficient synthesis of β(1,4)-oligo- and polysaccharides using a mutant cellulase, *J. Am. Chem. Soc. 122*, 5429−5437.
17. Hrmova, M., Imai, T., Rutten, S. J., Fairweather, J. K., Pelosi, L., Bulone, V., and Driguez, H. (2002) Barley 1,3-β-D-glucan endohydrolase mutants synthesise crystalline 1,3-β-D-glucans, *J. Biol. Chem. 277*, 30102−30111.
18. Jahn, M., Stoll, D., Warren, R. A. J., Szabo, L., Singh, P., Gilbert, H. J., Ducros, V. M., Davies, G. J., and Withers, S. G. (2003) Expansion of the glycosynthase repertoire to produce defined manno-oligosaccharides, *Chem. Commun.*, 1327−1329.
19. Planas, A. (2000) Bacterial 1,3-1,4-β-glucanases: structure, function and protein engineering, *Biochim. Biophys. Acta 1543*, 361−382.
20. Coutinho, P. M., and Henrissat, B. (1999) Carbohydrate-active enzymes: an integrated database approach, in *Recent Advances in Carbohydrate Bioengineering* (Gilbert, H. J., Davies, G. J., Henrissat, B., and Svensson, B., Eds.) pp 3−12, The Royal Society of Chemistry, Cambridge, U.K.
21. Viladot, J. L., de Ramon, E., Durany, O., and Planas, A. (1998) Probing the mechanism of *Bacillus* 1,3-1,4-β-D-glucan 4-glucanohydrolases by chemical rescue of inactive mutants at catalytically essential residues, *Biochemistry 37*, 11332−11342.
22. Hahn, M., Pons, J., Planas, A., Querol, E., and Heinemann, U. (1995) Crystal structure of *Bacillus licheniformis* 1,3-1,4-β-D-glucan 4-glucanohydrolase at 1.8 Å resolution, *FEBS Lett. 374*, 221−224.
23. Heinemann, U., Aÿ, J., Gaiser, O., Müller, J. J., and Ponnuswamy, M. N. (1996) Enzymology and folding of natural and engineered bacterial β-glucanases studied by X-ray crystallography, *Biol. Chem. 377*, 447−454.
24. Faijes, M., Fairweather, J. K., Driguez, H., and Planas, A. (2001) Oligosaccharide synthesis by coupled endo-glycosynthases of different specificity: a straightforward preparation of two mixed-linkage hexasaccharide substrates of 1,3-1,4-β-glucanases, *Chem. Eur. J. 7*, 4651−4655.
25. Malet, C., Viladot, J. L., Ochoa, A., Gállego, B., Brosa, C., and Planas, A. (1995) Synthesis of 4-methylumbelliferyl-β-D-glucan oligosaccharides as specific chromophoric substrates of 1,3-1,4-β-D-glucan 4-glucanohydrolases, *Carbohydr. Res. 274*, 285−301.
26. Viladot, J. L., Moreau, V., Planas, A., and Driguez, H. (1997) Transglycosylation activity of *Bacillus* 1,3-1,4-β-D-glucan 4-glucanohydrolases. Enzymatic synthesis of alternate 1,3-1,4-β-D-glucooligosaccharides, *J. Chem. Soc., Perkin Trans. 1*, 2383−2387.
27. van Tilbeurgh, H., Claeyssens, M., and de Bruyne, C. K. (1982) The use of 4-methylumbelliferyl and other chromophoric glycosides in the study of cellulolytic enzymes, *FEBS Lett. 149*, 152−156.
28. Malet, C., Vallés, J., Bou, J., and Planas, A. (1996) A specific chromophoric substrate for activity assays of 1,3-1,4-β-D-glucan 4-glucanohydrolases, *J. Biotechnol. 48*, 209−219.
29. Planas, A., Millet, O., Palasí, J., Pallarés, C., Abel, M., and Viladot, J. L. (1998) Synthesis of aryl 3-*O*-β-cellobiosyl-β-D-glucopyranosides for reactivity studies of 1,3-1,4-β-glucanases, *Carbohydr. Res. 310*, 53−64.
30. Braman, J., Papworth, C., and Greener, A. (1996) Site-directed mutagenesis using double-stranded plasmid DNA templates, *Methods Mol. Biol. 57*, 31−44.
31. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
32. Lloberas, J., Querol, E., and Bernués, J. (1988) Purification and characterization of endo-β-1,3-1,4-D-glucanase activity from *Bacillus licheniformis*, *Appl. Microbiol. Biotechnol. 29*, 32−38.
33. Malet, C., and Planas, A. (1997) Mechanism of *Bacillus* 1,3-1,4-β-D-glucan 4-glucanohydrolases: kinetics and pH studies with 4-methylumbelliferyl β-glucan oligosaccharides, *Biochemistry 36*, 13838−13848.
34. Ellis, K. J., and Morrison, J. F. (1982) Buffers of constant ionic strength for studying pH-dependent processes, *Methods Enzymol. 87*, 405−426.
35. Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966) Tetranitromethane. A reagent for the nitration of tyrosyl residues in proteins, *Biochemistry 5*, 3582−3589.
36. Malet, C., Jiménez-Barbero, J., Bernabé, M., Brosa, C., and Planas, A. (1993) Stereochemical course and structure of the products of the enzymic action of endo-1,3-1,4-β-D-glucan 4-glucanohydrolase from *Bacillus licheniformis*, *Biochem. J. 296*, 753−758.
37. Bock, K., Pedersen, C., and Pedersen, H. (1984) Carbon-13 nuclear magnetic resonance data for oligosaccharides, *Adv. Carbohydr. Chem. Biochem. 42*, 193−225.
38. Bradbury, J. H., and Jenkins, G. A. (1984) Determination of the structures of trisaccharides by ^{13}C NMR spectroscopy, *Carbohydr. Res. 126*, 125−156.
39. Montero, E., Alonso, J., Canada, F. J., Fernandez-Mayoralas, A., and Martin-Lomas, M. (1997) Regioselectivity of the enzymatic transgalactosidation of D- and L-xylose catalysed by β-galactosidases, *Carbohydr. Res. 305*, 383−391.
40. Segel, I. H. (1975) in *Enzyme kinetics*, John Wiley and Sons, New York.
41. Hoare, D. G., and Koshland, D. E., Jr. (1967) A method for the quantitative modification and estimation of carboxylic acid groups in proteins, *J. Biol. Chem. 242*, 2447−2453.
42. Carraway, K. L., and Koshland, D. E., Jr. (1972) Carbodiimide modification of proteins, *Methods Enzymol. 25*, 616−623.
43. Levy, H. M., Leber, P. D., and Ryan, E. M. (1963) Inactivation of myosin by 2,4-dinitrophenol and protection by adenosine triphosphate and other phosphate compounds, *J. Biol. Chem. 238*, 3654−3659.
44. Cardemil, E. (1996) Chemical modification of enzymes: kinetic aspects, *Biol. Res. 29*, 13−20.
45. Carraway, K. L., and Tripplett, R. B. (1970) Reaction of carbodiimides with protein sulfhydryl groups, *Biochim. Biophys. Acta 200*, 564−566.
46. Carraway, K. L., and Koshland, D. E., Jr. (1968) Reaction of tyrosine residues in proteins with carbodiimide reagents, *Biochim. Biophys. Acta 160*, 272−274.
47. Hahn, M., Keitel, T., and Heinemann, U. (1995) Crystal and molecular structure at 0.16 nm resolution of the hybrid *Bacillus* endo-1,3-1,4-β-D-glucan 4-glucanohydrolase H(A16-M), *Eur. J. Biochem. 232*, 849−859.
48. Planas, A. (1998) Mutational analysis of specificity and catalysis in *Bacillus* 1,3-1,4-β-glucanases, in *Carbohydrases from Trichoderma reesei and other microorganisms* (Claeyssens, M., Nerinckx, W., and Piens, K., Eds.) pp 21−38, The Royal Society of Chemistry, Cambridge, U.K.
49. Fort, S., Christiansen, L., Schülein, M., Cottaz, S., and Driguez, H. (2000) Stepwise synthesis of cellodextrins assisted by a mutant cellulase, *Isr. J. Chem. 40*, 217−221.

50. Fairweather, J. K., Faijes, M., Driguez, H., and Planas, A. (2002) Specificity studies of *Bacillus* 1,3-1,4-β-glucanases and application to glycosynthase-catalyzed transglycosylation, *ChemBioChem 3*, 866−873.
51. McIntosh, L. P., Hand, G., Johnson, P. E., Joshi, M. D., Korner, M., Plesniak, L. A., Ziser, L., Wakarchuk, W. W., and Withers, S. G. (1996) The pK_a of the general acid/base carboxyl group of a glycosidase cycles during catalysis: A ^{13}C-NMR study of *Bacillus circulans* xylanase, *Biochemistry 35*, 9958−9966.
52. Chan, V. W. F., Jorgensen, A. M., and Borders, C. L. (1988) Inactivation of bovine thrombin by water-soluble carbodiimides: the essential carboxyl group has a pK_a of 5.51, *Biochem. Biophys. Res. Commun. 151*, 2−709.
53. Svensson, B., Clarke, A. J., Svendsen, I., and Moller, H. (1990) Identification of carboxylic acid residues in glucoamylase G2 from *Aspergillus niger* that participate in catalysis and substrate binding, *Eur. J. Biochem. 188*, 29−38.

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