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A transitional hydrolase to glycosynthase mutant by Glu to Asp substitution at the catalytic nucleophile in a retaining glycosidase

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ABSTRACT

Glycosynthases from more than 16 glycosidase families have been developed for the efficient synthesis of oligosaccharides and glycoconjugates. β-1,3-1,4-Glucan oligo- and polysaccharides with defined sequences can be quantitatively achieved with the glycosynthases derived from *Bacillus licheniformis* β-1,3-1,4-glucanase. The screening of a nucleophile saturation library of this enzyme yielded the unexpected E134D mutant which has high glycosynthase efficiency (25% higher k_{cat} than the best glycosynthase to date, E134S) but also retains some hydrolase activity (2% relative to the wild-type enzyme). Here, we report the biochemical and structural analyses of this mutant compared to E134S and wild-type enzymes. E134D shows a pH profile of general base catalysis for the glycosynthase activity, with a kinetic pK_a (on k_{cat}/K_M) assigned to Glu138 of 5.8, whereas the same residue acts as a general acid in the hydrolase activity with the same pK_a value. The pK_a of Glu138 in the wt enzyme was 7.0, a high value due to the presence of the catalytic nucleophile Glu134 which destabilizes the conjugate base of Glu138. Thus, the pK_a of Glu138 drops 1.1 pH units in the mutant relative to the wild-type enzyme meaning that the larger distance between carboxylates in positions 138 and 134 (5.6 Å for wt, 7.0 Å for E134D) and/or a new hydrogen bonding interaction with a third Asp residue (Asp136) in the mutant reduces the effect of the negatively charged Asp134. In consequence, the pK_a of Glu138 has a similar pK_a value in the E134D mutant than in the other glycosynthase mutants having a neutral residue in position 134. The behavior of the E134D mutant shows that shortening the side chain of the nucleophile, despite maintaining a carboxylate group, confers glycosynthase activity. Therefore E134D is a transitional hydrolase to glycosynthase mutation.

1. Introduction

Retaining glycosidases (or glycoside hydrolases, GH) catalyze the hydrolysis of glycosidic bonds of oligosaccharides, polysaccharides, and glycoconjugates by general acid/base catalysis in a double displacement reaction with net retention of the anomeric configuration. The catalytic mechanism involves two essential residues, a general acid/base and a catalytic nucleophile, the reaction proceeding through the formation of a covalent glycosyl-enzyme intermediate[1] (Fig. 1A). A variation of this mechanism is found in some hexosaminidases which operate by substrate-assisted catalysis; they lack the catalytic nucleophile and the 2-acetamido group of the substrate acts as an internal nucleophile.[2,3] Retaining GH have been extensively used for enzymatic synthesis of oligosaccharides and glycoconjugates through their ability to catalyze transglycosylation reactions.[4] However, kinetically-controlled transglycosylation is severely hampered by the predominant hydrolase activity, where the product formed is necessarily a hydrolysable substrate. The introduction of the glycosynthases concept in 1998[5,6] overcomes this limitation for the efficient enzymatic synthesis of oligosaccharides and glycoconjugates.

Glycosynthases are engineered retaining glycoside hydrolases in which the catalytic nucleophile has been replaced by a non-nucleophilic residue. They are inactive hydrolases but efficiently catalyze glycosyl transfer to an acceptor when using activated glycosyl fluoride donors with the opposite anomeric configuration than the original substrate of the parental wt hydrolase reaction (Fig. 1B). Enzymes from more than 16 different GH families (according to the CAZY database classification[7]) have been converted into glycosynthases. They cover different specificities and relevant applications have been developed (for reviews see[8–11]).

Bacillus licheniformis 1,3-1,4-β-glucanase is a retaining *endo*-glycosidase belonging to CAZY family GH16, which hydrolases mixed-linked 1,3-1,4-β-glucans.[12] The catalytic machinery involves a catalytic triad formed by Glu138 and Glu134 as general

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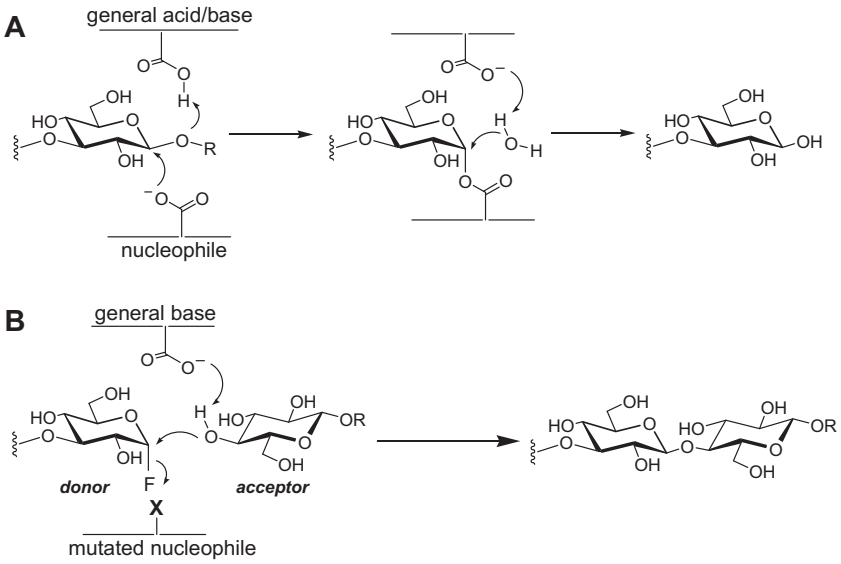

Figure 1. (A) Mechanism of a retaining glycosidase involving the participation of a general acid/base residue and a catalytic nucleophile residue. (B) Mechanism of a glycosynthase mutant, where X is a non-nucleophilic substitution for the original nucleophile in the wt enzyme.

acid/base and catalytic nucleophile, respectively, and Asp136 as assisting residue that participates in the modulation of the pK_as of the catalytic residues.[13,14] Nucleophile mutants E134A and E134S are efficient glycosynthases catalyzing condensation reactions between glycosyl fluoride donors of general structure (Glcβ4)nGlcβ3GlcαF and gluco-oligosaccharide acceptors with regioselective formation of β-1,4 glycosidic bonds.[6,15–18] More recently, they have shown to be efficient catalysts for the preparation of artificial polysaccharides with regular structures through glycosynthase-catalyzed donor self-condensation.[19]

During the development of a screening assay for the analysis of mutant glycosynthase libraries (HTS based on a fluoride chemosensor assay),[20] we analyzed a nucleophile saturation library (E134X) of the *Bacillus licheniformis* 1,3-1,4-β-glucanase. In addition to the expected and well known glycosynthase variants (Ala, Ser, and Gly mutations), a surprising result was the finding that E134D showed high glycosynthase activity while keeping residual hydrolase activity. Shortening the side chain of the nucleophile residue (Glu to Asp) was already known to decrease significantly the hydrolase activity of β-glycosidases (e.g. lacZ β-galactosidase,[21] *Agrobacterium* β-glucosidase,[22] *B. circulans* xylanase[23]), but this conservative substitution has not been previously reported as a glycosynthase. This surprising result may be interpreted as a transitional form between the original hydrolase and its glycosynthase variants. Here we analyze the properties of the E134D mutant 1,3-1,4-β-glucanase with the aim of understanding how a conservative mutation (Asp for Glu substitution) still maintaining a carboxylate group behaves as a glycosynthase. pH studies reveal significant aspects about the modulation of the pK_as of the catalytic residues.

2. Material and methods

2.1. Protein expression and purification

All the proteins (wt, E134A, E134S, and E134D) were cloned in a pET16b vector as reported.[20,24] *Escherichia coli* BL21 DE3 Star cells harboring the corresponding expression plasmid were grown in 500 mL LB medium (100 mg/mL ampicillin, 2% glucose) for 8 h at 37 °C and 250 rpm until late exponential phase. The medium was changed to 500 mL LB containing 1 mM IPTG and 100 mg/mL ampicillin for protein expression (16 h at 25 °C). Cells were harvested by centrifugation and resuspended in 100 mL phosphate buffer 50 mM, pH 7.0, 0.1 mM $CaCl_2$. Proteins were purified essentially as reported for the WT enzyme by means of metal affinity chromatography of the His-tagged proteins.[24] Cells were lysed in a cell-disrupter (Constant Systems, UK) and centrifuged at 12,000 rpm. The supernatant was loaded onto a HiTrap 1 mL column (GE Healthcare) previously equilibrated with loading buffer (50 mM phosphate pH 7.0, 0.1 mM $CaCl_2$). The column was rinsed to remove unbound proteins and then eluted with a gradient of 0.5 M imidazole in 50 mM phosphate pH 7.0, 0.1 mM $CaCl_2$. Protein fractions were dialyzed twice against 50 mM phosphate pH 7.0, 0.1 mM $CaCl_2$, followed by a last dialysis against water. Proteins were lyophilized for storage, and redissolved prior to use. Enzymes were >95% homogeneous as judged by SDS–PAGE. Concentration was determined by absorbance at 280 nm using an extinction coefficient of $3.53{\cdot}10^5\ M^{-1}\ cm^{-1}$[25].

2.2. Kinetics of glycosynthase reactions

The glycosyl donor and acceptor, β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-α-D-glucopyranosyl fluoride (Glcβ4Glcβ3GlcαF)[25] and *p*-nitrophenyl β-D-glucopyranoside (GlcβpNP) respectively, were dissolved in phosphate–citrate buffer (50 mM citric acid and 50 mM Na_2HPO_4), $CaCl_2$ (0.1 mM) at the indicated pH, and with a constant ionic strength of $I = 0.5$ M adjusted with KCl. The solution was preincubated at 35 °C for 5 min. Reactions were initiated by addition of the correspondent enzyme and the mixtures kept at 35 °C. Aliquots were taken at different time intervals and diluted 1:10 in 2% (v/v) formic acid to stop the enzymatic reaction. HPLC analysis was done in an Agilent HPLC 1100 chromatograph using a reverse phase column (NovaPak C18, 4 μm, 3.9 mm × 150 mm, Waters), eluted with 12–14% MeOH in H_2O at a flow of 1 mL/min, UV detector at 310 nm. Initial rates (v_0) were calculated from the linear portion of the progress curves (normalized area vs time) and expressed as $v_0/[E]{\cdot}(s^{-1})$.

For the reaction progress curve, mutants E134D and E134S were assayed at 2 μM enzyme concentration, 2 mM donor and 20 mM acceptor in phosphate buffer (50 mM pH 7 and $CaCl_2$ 0.1 mM) at 35 °C. The formation/disappearance of glycosynthase-derived products was followed by HPLC as described above.

The pH dependence of the glycosynthase activity was determined on k_{cat} and k_{cat}/K_M in the pH range 5 to 9. At each pH, donor was varied (0.1 to 8 mM, 11 different concentrations) and acceptor

kept constant at 20 mM. Initial rates were fitted to the Michaelis–Menten equation to calculate the kinetic parameters by non-linear regression. For E134D, k_{cat}/K_M versus pH data were fitted to an upward single ionization curve (Eq. 1).

$$\frac{k_{cat}}{K_M} = \frac{A}{1 + 10^{(pK_a - pH)}}. \quad (1)$$

For E134S, k_{cat}/K_M versus pH data were fitted to a double ionization curve with residual activity at high pH, as reported for the E134A glycosynthase mutant[25] (Eq. 2).

$$\frac{k_{cat}}{K_M} = \frac{A + B \cdot 10^{(pH - pK_{a2})}}{1 + 10^{(pK_{a1} - pH)} + 10^{(pH - pK_{a2})}}. \quad (2)$$

2.3. Kinetics of hydrolase reactions

The hydrolase activity was determined with 4-methylumbelliferyl 3-*O*-β-cellobiosyl-β-D-glucopyranoside (Glcβ4Glcβ3GlcβMU) as substrate and following changes in UV absorbance due to the release of 4-methylumbelliferone (MU) using matched 1 cm path length cells at 35 °C in a Evolution 300 spectrophotometer (Thermo Scientific).[13] Rates of the enzyme-catalyzed reactions were determined by incubating the enzyme with the substrate in citrate/phosphate buffer (50 mM citric acid and 50 mM Na_2HPO_4) and 0.1 mM $CaCl_2$ in the pH range of 5–7 in the thermostated cell holder. Reactions were initiated by addition of enzyme to the pre-incubated mixture of substrate and buffer. The absorbance change was monitored at a wavelength of $\lambda = 365$ nm. Extinction coefficients for MU at 365 nm were determined at each pH under the same experimental conditions. Initial rates (v_0) were calculated from the linear portion of the progress curves. For wt, the slope of v_0 versus substrate concentration (0.1–0.3 mM) gave the k_{cat}/K_M values at each pH. k_{cat}/K_M versus pH data were fitted to a bell-shaped double ionization curve (Eq. 3)

$$\frac{k_{cat}}{K_M} = \frac{A}{1 + 10^{(pK_{a1} - pH)} + 10^{(pH - pK_{a2})}}. \quad (3)$$

For E134D, initial rates were determined at 1 mM substrate concentration at each pH. These rates at low substrate concentration were assumed to reflect k_{cat}/K_M. Hydrolysis rates at lower substrate concentrations (to determine true k_{cat}/K_M values from the linear dependence v_0 vs [*S*]) were difficult to measure due to the low hydrolase activity of the mutant. $v_0/[E]$ ($\approx k_{cat}/K_M$) versus pH data were fitted to a downward single ionization curve (Eq. 4)

$$\frac{v_0}{[E]} = \frac{A}{1 + 10^{(pH - pK_a)}}. \quad (4)$$

All fittings were done with the FigP software. Estimated standard errors in pK_a determinations were ±0.1 pH units.

2.4. Chemical rescue

This assay was performed following a modification of the procedure reported in Viladot et al.[26] A solution of enzyme (E134A, E134S, or E134D) (1 μM) and sodium azide (1 M) was preincubated in a thermostated cell holder at 30 °C in phosphate–citrate buffer (87 mM phosphate, 6.5 mM citrate) pH = 7.2. Reactions were initiated by adding 2,4-dintrophenyl 3-*O*-β-cellobiosyl-β-D-glucopyranoside (Glcβ4Glcβ3GlcβDNP) substrate (1 mM), and the release of 2,4-dinitrophenol (DNP) was monitored by absorbance changes at 425 nm, using an extinction coefficient of $\Delta\varepsilon$ 6134 M^{-1}-cm^{-1}.[26,27] Blank reactions without sodium azide were run in parallel to substract the background enzymatic hydrolysis rate of this reactive substrate. Initial rate was calculated as the slope of DNP concentration (reaction-blank) versus *t*.

2.5. Enzymatic polymerization

Reactions were carried out in phosphate buffer (50 mM pH 7, 0.1 mM $CaCl_2$). The donor substrate Glcβ4Glcβ3GlcαF (150 mM) was incubated with different enzyme concentrations at 35 °C with orbital shaking at 250 rpm for 24 h. Enzyme concentrations for E134S and E134D were chosen as to have the same activity according to the higher k_{cat} value for E134D: 19 μM (E134D), 25 μM (E134S) for low enzyme concentration, and 47 μM (E134D), 63 μM (E134S) for high enzyme concentration. A precipitate was formed during the reactions. It was recovered by centrifugation at 13,000 rpm for 5 min, washed several times with cold water, and freeze-dried. Product yields are expressed in % (weight polymer/weight initial donor) for the insoluble material. Supernatants were also lyophilized to recover soluble oligomers. Products were analyzed by HPSEC to determine the molecular mass profiles as described in Perez et al.[19] Analyses were performed on an Agilent 1100 HPLC system equipped with a refractive index detector (RID) using a PSS Gram column (8.0 × 300 mm, 100 Å, 10 μm) and a PSS Gram precolumn (8.0 × 50 mm, 100 Å, 10 μm) thermostated at 50 °C, and DMSO with lithium bromide (5 g/L) as eluent at a flow rate of 0.5 mL/min. The calibration curve was obtained with dextrans as standards in the range 1–55 kDa (American Polymer Standards Corporation DXT1–DXT55 kDa). Freeze-dried polymers from the enzymatic reactions and standards were dissolved in DMSO and filtered. From the chromatograms, Mp (molecular mass of the peak maximum), Mw (weight average molecular mass), Mn (number average molecular mass), DP (degree of polymerization), and PDI (polydispersity index) were calculated.[28]

2.6. Modeling of the enzyme·substrate complexes

The three dimensional structures of different enzyme·substrate complexes of *Bacillus licheniformis* 1,3-1,4-β-glucanase were modeled in silico by means of homology modeling.[29] The structure of the wt·substrate complex was previously modeled.[30] E134S and E134D variants were modeled based on the 3D structure of the free enzyme (PDB accession code: 1GBG). The substrate was extracted from the structure of the orthologous *Bacillus macerans* 1,3-1,4-β-glucanase in complex with the tetrasaccharide product (Glcβ4Glcβ4Glcβ3Glc) (PDB accession code: 1U0A), which was manually converted to an α-fluoride glycosyl donor (Glcβ3GlcαF), and its position in the structure was kept fixed during the modeling. 100 replicas of the models were generated for each variant, in which different orientations of the side-chains were sampled during a brief simulated annealing and geometry optimization process.

3. Results

The ability of the E134D 1,3-1,4-β-glucanase to behave as a glycosynthases poses a number of questions about the mechanism: how large is the cavity created by shortening the Glu to Asp side chain to accommodate the α-fluoride of the donor substrate?; does the mutant enzyme operate by general base catalysis thus implying a large downward shift of the pK_a of E138 (general acid in the wt hydrolase mechanism) to behave as a base in the glycosynthase mechanism?; which is the extent and pH dependence of the residual hydrolase activity?; is it a practical mutant for the enzymatic synthesis of polysaccharides by donor self-condensation?.

3.1. Glycosynthase activity of mutant E134D

The time course of the glycosynthase reaction of E134D at pH 7, 35 °C, is compared to that of the well characterized E134S

glycosynthase using Glcβ4Glcβ3GlcαF (2 mM) as donor and GlcβpNP (20 mM) as acceptor substrates, where the acceptor is in large excess to minimize donor self-condensation. As shown in Figure 2, E134S rapidly forms the condensation product (Glcβ4Glcβ3Glcβ4GlcβpNP) up to 90% yield, and the product remains stable upon long incubation time. E134D has the same initial reaction rate, reaching a maximum of 90% yield, but the product is then slowly hydrolyzed due to the still significant hydrolase activity. Kinetic parameters had been previously determined[20] and are summarized in Table 1. Under initial rate conditions (<10% conversion), E134D has 25% higher k_{cat} than E134S and E134A, but K_M (donor) is in between both Ser and Ala glycosynthase mutants.

The pH dependence of the glycosynthase activity for E134D, compared to that of E134S, is presented in Figure 3. The E134D mutant shows a pH dependence corresponding to general base catalysis, with a kinetic pK_a (on k_{cat}/K_M) of 5.8 and maximum activity at pH 7. At pH values higher than 7.5 the protein is less stable and precipitates, precluding kinetic measurements. The profile up to pH 7 is similar to that of E134S (and the previously reported E134A mutant,[17] with the same kinetic pK_a than E134S (5.8) and slightly higher than E134A (5.2). It corresponds to Glu138 acting as general base as it was previously assigned in the E134A mutant by titration with a water-soluble carbodiimide.[25]

3.2. Hydrolase activity of E134D

The hydrolase activity of E134D was determined with Glcβ4Glcβ3GlcβMU (MU: 4-methylumbelliferyl), a common β-aryl glycoside substrate of the wt enzyme, by monitoring MU release. At pH 7, 35 °C, the specific activity of E134D is about 2% of the wt enzyme. To evaluate the kinetic pK_a of the general acid residue E138 in the mutant, the pH dependence of the hydrolytic reaction was determined and compared to that of the wt enzyme. For the wt enzyme, the pH profile of k_{cat}/K_M (Fig. 4A) follows a double ionization curve, with a high pK_a of 7.0 corresponding to the general acid E138, and a low pK_a of 5.4 for the nucleophile E134. These values are close to those previously reported at 53 °C (pK_as of 7.0 and 5.5, respectively), temperature of maximum activity for the wt enzyme.[13] For E134D, rates at 1 mM substrate were assumed to reflect k_{cat}/K_M values (hydrolysis rates at lower substrate concentration were difficult to measure due to the low activity of the mutant). As shown in Figure 4B, the hydrolase activity decreases from pH 5 to 7, with a kinetic pK_a of 5.9 assignable to the general acid E138 in the hydrolase mechanism. Thus the E134D mutation results in a pK_a decrease of one pH unit. It is also consistent with the pK_a (5.8) determined for the glycosynthase activity and assigned to Glu138, but acting as general base (see Section 4).

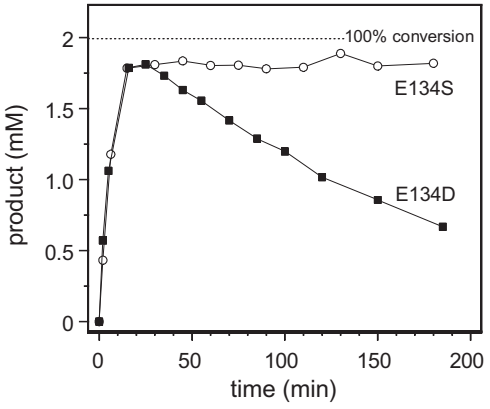

Figure 2. Time-course monitoring of the glycosynthase reactions catalyzed by the E134D and E134S mutants. Conditions: 2 mM Glcβ4Glcβ3GlcαF donor, 20 mM GlcβpNP acceptor, 2 μM enzyme, pH 7.0, 35 °C.

3.3. Chemical rescue with an exogenous nucleophile

Alanine mutants at the catalytic nucleophile can be reactivated using an exogenous nucleophile that can bind to the cavity left by removal of the catalytic carboxylate (Ala for Glu mutation). The E134A 1,3-1,4-β-glucanase is reactivated by addition of azide in a concentration dependent manner using an activated 2,4-dinitrophenyl β-glycoside substrate, to give the α-glycosyl azide product.[26,31,32] The experiment was applied to the E134S and E134D mutants by incubating the enzymes with 1 mM Glcβ4Glcβ3GlcβDNP (DNP: 2,4-dinitrophenyl) and 1 M sodium azide at pH 7.2 and 30 °C. The release of DNP (after subtracting the blank rate in the absence of sodium azide) is plotted in Figure 5. E134S shows reactivation (*chemical rescue*) but to a lesser extent than the E134A mutant, whereas the E134D mutant is not reactivated but instead, azide inhibits the residual hydrolase activity of the mutant.

3.4. Glycosynthase-catalyzed polymerization

The glycosynthase mutants E134A and E134S of 1,3-1,4-β-glucanase are able to produce artificial polysaccharides by donor self-condensation in the absence of an acceptor.[19,33] The E134S-catalyzed polymerization of the tetrasaccharide donor Glcβ4Glcβ4Glcβ3GlcαF led to high molecular mass polysaccharides (up to Mw of 30 kDa), where the degree of polymerization (DP) was linearly dependent on enzyme activity. Since the polymers are water-insoluble at rather low Mw, it was hypothesized that the balance between enzymatic polymerization rate and polymer precipitation rate dictates the molecular mass of the products.[19] In keeping with that, a more active glycosynthase would in principle achieve polysaccharides with higher DP. Although the new E134D mutant is not a practical glycosynthase for soluble oligosaccharide synthesis due to the residual hydrolase activity, it might be efficient for polysaccharide synthesis: insoluble polysaccharides might be formed fast (E134D has a 25% higher k_{cat} than E134S (Table 1)) and their precipitation protects them from hydrolysis.

Polymerization reactions catalyzed by E134S and E134D were performed with the trisaccharide donor Glcβ4Glcβ3GlcαF (150 mM) in phosphate buffer pH 7 at 35 °C. Precipitated polysaccharides and the soluble fractions were analyzed by HPSEC (Fig. 6). Results are summarized in Table 2. For E134S (at low and high concentrations) the insoluble polymers show a monomodal distribution of molecular masses with Mw of 13 kDa, (DP 78), corresponding to 26 condensations, whereas the soluble fraction only contains traces of oligosaccharides. For E134D the size distribution of the precipitated polymers is multimodal and dependent of enzyme concentration. At low (19 μM) concentration, an insoluble polymer with Mw 5.5 kDa is obtained, with a main peak (Mp) at 5.3 kDa (DP 33), and a minor peak at Mp 0.6 kDa for a trisaccharide, either unreacted donor or hydrolysis product. At higher enzyme concentration (47 μM) Mw increases up to 14 kDa, and the molecular mass distribution becomes trimodal with Mp 32.7, 4.7, and 0.6 kDa, corresponding to DP 201, 30, and 3, respectively. A significant fraction of a larger polymer is obtained, but some trisaccharide (Mp 0.6 kDa) is still present. As opposed to the case of E134S, the soluble fractions in the E134D reactions contain a significant amount of trisaccharide (Mp 0.6 kDa) and hexasaccharide (Mp 1.2 kDa) at low enzyme concentration and mainly trisaccaride at high enzyme concentration. The nature of these small molecules may be explained by two non-excluding hypotheses: unreacted donor or hydrolysis of the longer polymers formed. In agreement with the progress curve of the glycosynthase reaction shown in

Table 1
Kinetic parameters for the glycosynthase activity of E134D, S, and A mutants

	k_{cat} (s^{-1})	$K_{M,donor}$ (mM)	$(k_{cat}/K_M)_{donor}$ (M^{-1} s^{-1})	% k_{cat}	% k_{cat}/K_M
E134D	1.44 ± 0.05	0.49 ± 0.05	$2.95 \cdot 10^3$	100	27
E134S	1.08 ± 0.04	0.10 ± 0.02	$1.08 \cdot 10^4$	75	100
E134A	1.04 ± 0.04	1.12 ± 0.12	$9.27 \cdot 10^2$	72	9

Conditions: 0–8 mM donor (Glcβ4Glcβ3GlcαF), 20 mM acceptor (Glc-pNP), 0.5 μM enzyme, 50 mM phosphate pH 7.0, 0.1 mM $CaCl_2$, 35 °C.

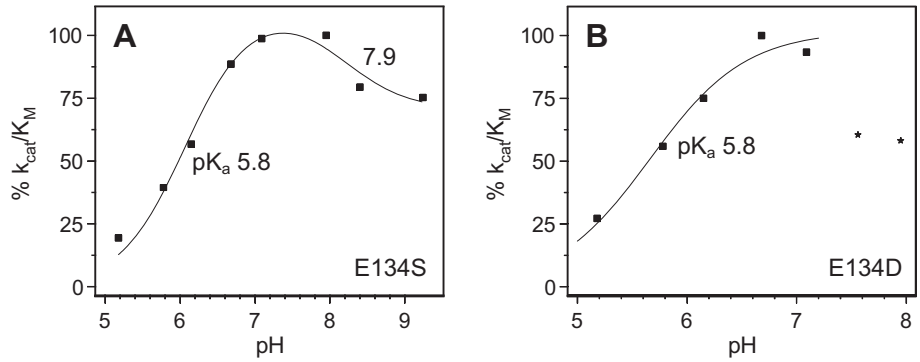

Figure 3. pH dependence of k_{cat}/K_M for the glycosynthase activity of E134S (A) and E134D (B) mutant enzymes. At pH >7.5, E134D activity decreases due to protein precipitation (*in the graph).

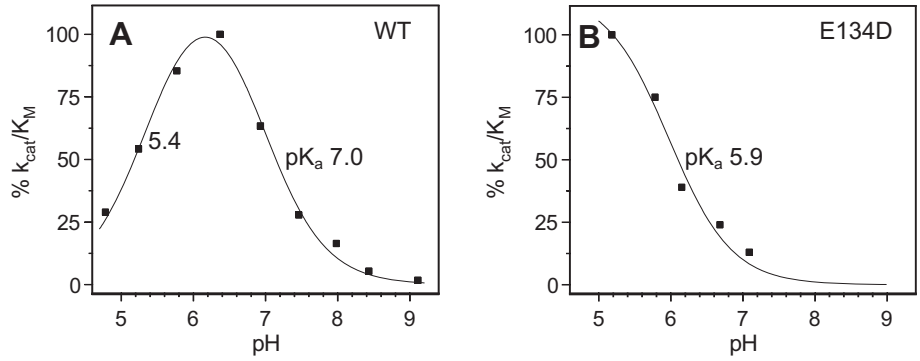

Figure 4. pH dependence of k_{cat}/K_M for the hydrolytic activity of the WT (A) and E134D mutant (B) enzymes.

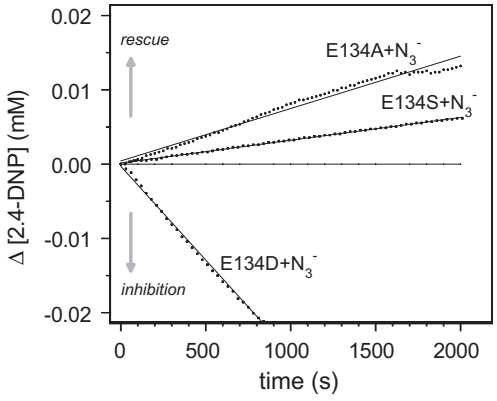

Figure 5. Chemical rescue by exogenous azide of mutants E134A, E134S, and E134D. Conditions: 1 mM Glcβ4Glcβ3GlcβDNP, 1 M NaN_3, 1 μM enzyme, citrate–phosphate buffer pH 7.2, 0.1 mM $CaCl_2$, 30 °C.

Figure 2 (high synthase activity but then product hydrolysis), it is more likely that they are hydrolysis products. Moreover, the yield of insoluble polymers was low (10–20%) for the E134D reactions (about 90% for E134S), and only a trisaccharide product was observed in the soluble fraction at high enzyme concentration, all supportive of hydrolysis of the products formed.

3.5. Modeling the enzyme·substrate complex in the glycosynthase mutants

Mutants E134D and E134S with bound α-glycosyl fluoride substrate were modeled in silico and compared to the modeled Michaelis complex of the wt 1,3-1,4-β- glucanase previously reported[30,34] (Fig. 7). A β-glycoside substrate binds with a $^1S_3$ distorted conformation in the Michaelis complex of the wt enzyme (preactivated conformation for catalysis in the hydrolase mechanism[30,35]), whereas the α-glycosyl fluoride donor in the glycosynthase mutants remains in a chair ($^4C_1$) conformation (as recently observed in the X-ray 3D structures of glycosynthase mutants of rice BGlu1[36]). The shorter side chain of the mutants at position 134 relative to the wild-type creates a cavity that accommodates the fluoride aglycon with α-configuration. The distance between the carboxylates in position 138 and 134 is 5.6 Å in the wt, and 7.0 in the E134D mutant. For the Ser mutant (Fig. 7C), the hydroxyl oxygen is at approximately 4.3 Å of the fluoride atom, with an orientation that may allow for a weak hydrogen bonding interaction to assist the departure of the fluoride aglycon in the SN_2 displacement reaction by the acceptor. For the Asp mutant

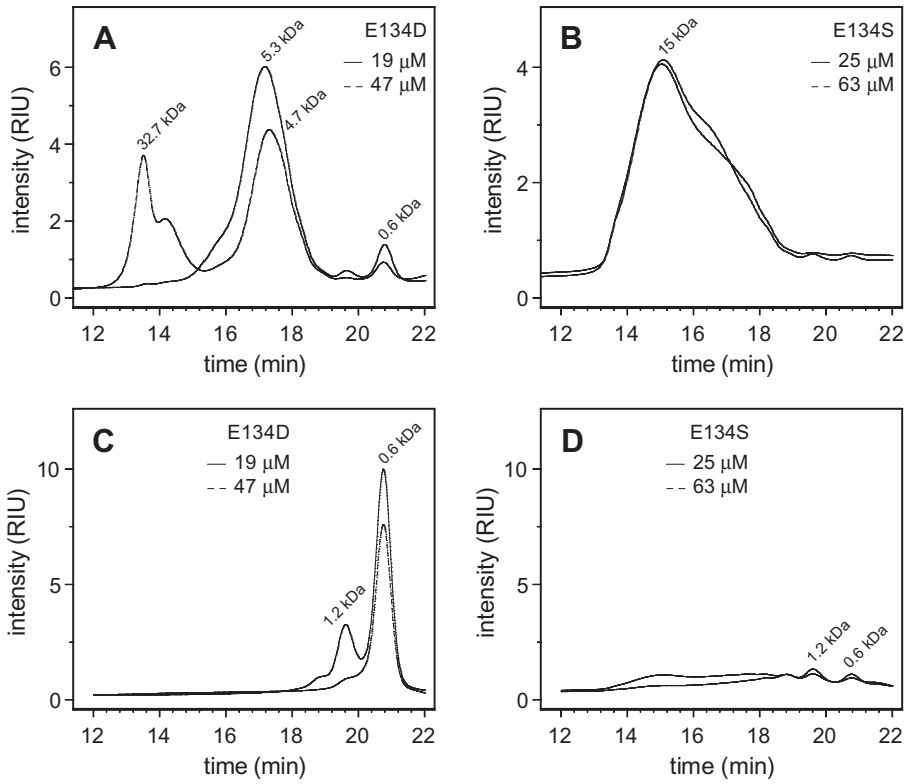

Figure 6. Size exclusion chromatograms (HPSEC) of the insoluble polysaccharides by glycosynthase-catalyzed polymerization, (A) with E134D, (B) with E134S, and of the soluble fractions, (C) with E134D, (D) with E134S, at two different enzyme concentrations. Mp values (maximum of the peaks) are indicated in kDa.

Table 2
Enzymatic polymerization by E134D and E134S glycosynthase mutants

Mutant	[*E*] (μM)	Sample	Yield (%)	M_w (kDa)	DP[a]	M_n (kDa)	PDI	M_p[b] (kDa)
E134D	19	Precipitate	10	5.5	34	2.8	1.9	4.8, 0.6
		Supernatant	90	1.0	6	0.7	1.4	0.6, 1.2
	47	Precipitate	20	14.0	86	3.5	4.0	37.6, 4.4, 0.6
		Supernatant	80	1.7	10	0.8	2.3	0.6
E134S	25	Precipitate	80	12.3	76	4.2	2.9	15.8
		Supernatant	20	7.6	47	1.8	4.2	—
	63	Precipitate	90	12.4	76	4.4	2.8	15.4
		Supernatant	10	4.9	30	1.3	3.7	—

Reaction conditions were 50 mM phosphate buffer pH 7, 0.1 mM $CaCl_2$, 150 mM Glcβ4Glcβ3GlcαF donor, at 35 °C for 24 h.
[a] Degree of polymerization (DP) expressed as the number of glucosyl units for the weight average molecular mass polysaccharide (Mw).
[b] Mp, molecular mass of the peak maximum.

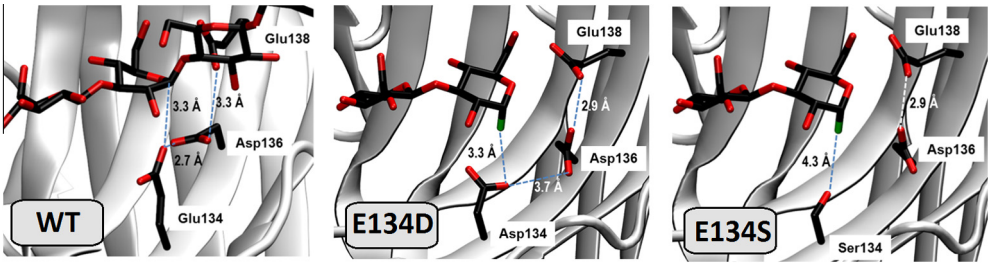

Figure 7. Modeled enzyme·substrate complexes, WT with Glcβ4Glcβ3Glcβ4Glc, E134D and E134S with Glcβ3GlcαF. Dashed lines are distances between heteroatoms expressed in Å.

(Fig. 7B), the distance between the fluoride and the carboxylate is shorter, and may involve a halogen bonding interaction (distance F···O of 3.3 Å and a C1–F···O angle of ≈160°, a geometry that perfectly fits that of halogen bonding interactions found in protein·ligand complexes).[37] Preliminary computational results by ab initio methods indicate that this type of electrostatic interaction, although present in α-halogen-glycosyl systems, is very weak for fluoride (data not shown).

It is relevant, as it will be discussed below, that Asp136, a third carboxylate of the catalytic triad in the wt enzyme, is hydrogen bonding to the nucleophile Glu134 (O···O distance of 2.7 Å, Fig. 7A) but the same residue is predicted by the modeling to hydrogen bond with Glu138 in the E134D and E134S mutants (Fig. 7B and C).

4. Discussion

Typical glycosynthases arise from replacement of the catalytic nucleophile of a retaining glycosidase by a non-nucleophilic residue. Surprisingly, the conservative Glu to Asp substitution (E134D mutant) in 1,3-1,4-β-glucanase results in an active glycosynthase. It was an unexpected result because the carboxylate group is still present although in a different position due to the shorter side chain. Two essential aspects of the glycosynthase mechanism are that the nucleophile mutation has to create room to allow binding of the donor with the fluoride aglycon in α, and that general base catalysis is required to activate the acceptor substrate.

Replacement of the catalytic nucleophile by Ala, Gly, or Ser in typical glycosynthases, results in a shorter side chain that creates a cavity for the accommodation of the α-fluoride of the donor. It can be tested by chemical rescue of the hydrolytically inactive mutant by addition of an exogenous nucleophile as sodium azide or formate. For the E134A mutant, addition of azide restores the activity with an activated β-glycoside substrate (such as 2,4-dinitrophenyl β-glycoside). Azide is able to bind into the cavity left by removal of the nucleophile side chain and displace the β-aglycone by an S_N2 mechanism to give the α-glycosyl azide product.[26] Whereas activity is restored in the E134A and E134S mutants, azide inhibits the hydrolase activity of the E134D mutant (Fig. 5). This behavior is similar to that observed with the wt enzyme.[26] It indicates that the Glu to Asp mutation does not leave enough room for azide to bind and act as a nucleophile, or that the negative charge of the Asp residue prevents proper binding of azide by electrostatic repulsion, resulting in any case in the absence of chemical rescue. Although this experiment does not prove or disprove that a cavity has been created, the fact that the mutant has glycosynthase activity confirms that the α-fluoride is bound. As shown in the modeled structure (Fig. 7B), the α-glycosyl fluoride is properly bound and oriented for catalysis.

According to the general mechanisms depicted in Figure 1, the residue acting as general acid in the first step of the hydrolase mechanism behaves as a base to activate the acceptor in the glycosynthase mechanism. This change of the pK_a of the same residue is the result of different environments in the wt and glycosynthase mutant due to the presence or absence of the catalytic nucleophile. In the wt 1,3-1,4-β-glucanase, Glu138 is the general acid with a pK_a of 7.0 (Fig. 4A) in the first step leading to the covalent glycosyl-enzyme intermediate in the hydrolase mechanism.[6] This high pK_a is in part due to the presence of the negatively charged catalytic nucleophile Glu134 which destabilizes the conjugate base of Glu138. It drops in the glycosyl-enzyme intermediate due to neutralization of the negative charge in the covalent complex allowing Glu138 to act as general base in the deglycosylation step leading to the hydrolysis product. This pK_a cycling of the general acid/base residue in retaining glycosidases has been nicely demonstrated in a *B. circulans* xylanase by ^{13}C-NMR titration.[38] In the glycosynthase mechanism catalyzed by the original E134A mutant, Glu138 has a lower pK_a of 5.2[25] because of the Ala for Glu134 replacement, where removal of the carboxylate group deletes the negative charge that destabilized the conjugate base of Glu138. The active site charge is equivalent to that in the covalent glycosyl-enzyme intermediate of the wt enzyme, thus Glu138 having a low pK_a. The same concept applies to the other glycosynthase variants having a neutral residue in the position of the original nucleophile, as it is the case of the E134S mutant (with a pK_a of 5.8, Fig. 3A). But what about the E134D mutation?

Glu138 should be able to act as a base to explain that the E134D mutant behaves as a glycosynthase. Certainly, the E134D mutant shows a pH dependence corresponding to general base catalysis, with a kinetic pK_a of 5.8 and maximum activity at pH 7 (Fig. 3B). This pK_a value is the same as that for E134S (5.8) and slightly higher than that for E134A (5.2). It is also consistent with the pH profile of the residual hydrolase activity of the mutant, with a calculated pK_a of 5.9 in the downward profile for general acid catalysis. Therefore, E134D can be seen as a transitional hydrolase to glycosynthase mutant depending on pH, with hydrolase activity at low pH and glycosynthase activity at high pH.

Possible reasons for the low pK_a of Glu138 in the E134D mutant are as follows: (a) the distance between the carboxylates in positions 138 and 134 is longer in the mutant than in the wt (Fig. 7), thus reducing the electrostatic effect of the carboxylate of Asp134 and resulting in a lower destabilization of the conjugate base of Glu138. As a consequence its pK_a becomes similar to that in the E134S and E134A neutral nucleophile mutations. (b) The carboxylate of Glu138 is stabilized by a H-bonding interaction with Asp136, the third auxiliary residue of the catalytic triad in family GH16 which participates in modulating the pK_as of the catalytic residues.[14] As observed in the X-ray structures of the wt enzyme, Asp136 is hydrogen bonding with Glu134 in the free enzyme,[39,40] but it is rearranged in a covalent enzyme–ligand complex to hydrogen bond with Glu138, and occupies an intermediate position in the enzyme–product complex.[41,42] Given the flexibility of the side chain of Asp136 (as observed in the crystal structures of the wt enzyme), the modeled structure of the E134D·substrate complex (Fig. 7B) proposes an orientation of the Asp136 side chain that can establish a H-bond with Glu138. Therefore, it is reasonable that, even with Asp134 being deprotonated, the carboxylate of Glu138 might be stabilized by Asp136, thus lowering its pK_a and being able to act as a base in the glycosynthase reaction.

Because E134D has a higher k_{cat} than E134S in the glycosynthase reaction (Table 1), it was tested in polymerization reactions. The question was whether the fast donor condensation leads to polymeric products that precipitate and become inaccessible to the enzyme before they are hydrolyzed by the residual hydrolase activity of the mutant. It is shown not to be the case since low yields in polymeric products were obtained as compared to the E134S mutant (Table 2). The polymers obtained (although in low amount) had a different distribution but were not significantly larger than those produced by the E134S glycosynthase. Although this new mutant is a more active glycosynthase in terms of initial rates, it is not practical as biocatalyst.

5. Conclusions

The screening of a nucleophile saturation library revealed that the E134D mutant is a novel glycosynthase. Characterization of the glycosynthase and residual hydrolase activities of this mutant allow to conclude that shortening the side chain of the residue at position 134 creates enough room for binding the α-fluoride donor, and that Glu138 has a low pK_a to act as a general base in the glycosynthase mechanism similar to other neutral substitutions in position 134. E134D represents a transition between a glycosidase and a glycosynthase, with hydrolase activity at low pH and synthase activity at high pH.

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