Long-lived glycosyl-enzyme intermediate mimic produced by formate re-activation of a mutant endoglucanase lacking its catalytic nucleophile

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The mutant E134A 1,3-1,4- β -glucanase from *Bacillus licheniformis*, in which the catalytic nucleophilic residue has been removed by mutation to alanine, has its hydrolytic activity rescued by exogenous formate in a concentration-dependent manner. A long-lived α -glycosyl formate is detected and identified by ¹H-NMR and matrix-assisted laser desorption ionization–time-of-flight-MS. The intermediate is kinetically competent, since it is, at least partially, enzymically hydrolysed, and able to

act as a glycosyl donor in transglycosylation reactions. This transient compound represents a true covalent glycosyl-enzyme intermediate mimic of the proposed covalent intermediate in the reaction mechanism of retaining glycosidases.

Key words: chemical rescue, covalent intermediate, glycosidase mechanism, glycosynthase, 1,3-1,4- β -glucanase.

INTRODUCTION

Glycosyl hydrolases are key enzymes in the degradation of oligo- and poly-saccharides, and their glycoconjugates, in a large number of biological processes [1,2]. These enzymes are grouped into 82 families of related amino acid sequence [3,4], where all members of a family exhibit the same stereochemical outcome when the glycosidic bond is hydrolysed: inversion or retention of the anomeric configuration. Retaining glycosidases operate by a double-displacement mechanism (Scheme 1) in which a glycosyl-enzyme intermediate is formed (glycosylation step) and hydrolysed (deglycosylation step) in a general acid/base-catalysed process through oxocarbenium ion-like transition states.

Since Koshland proposed the double-displacement mechanism of retaining glycosidases in 1953 [5], the nature of the glycosyl-enzyme intermediate has been controversial. C-type lysozyme (hen egg white, EC 3.2.1.17) has been proposed to proceed through the stabilization of a long-lived carboxylate–oxocarbenium ion pair between a carboxy residue (Asp⁵²) and the anomeric carbon atom [6,7], whereas chitinases are believed to operate by substrate-assisted catalysis with participation of the carbonyl oxygen atom of the C-2 N-acetyl group of the substrate, since they lack the equivalent aspartic acid residue to stabilize the intermediate [8,9]. The more general (equatorial $\rightarrow$

equatorial) glycosidase mechanism as proposed by Koshland and elaborated upon by Sinnott [1,10] (Scheme 1) establishes that the intermediate is covalently bound to the catalytic nucleophile in the active site of the enzyme. The main supporting evidence for the involvement of a tetrahedral covalent glycosyl-enzyme intermediate comes from α -deuterium kinetic isotope effects, and trapping experiments with mechanism-based inhibitors.

The use of substrates for which the deglycosylation step is rate-determining allowed the measurement of direct α -deuterium kinetic isotope effects on the hydrolysis of the intermediate (e.g. *Escherichia coli* β -galactosidase [11], β -glucosidases [12,13], or the *Cellulomonas fimi* exo- β -1,4-glycanase Cex [14]). These effects all indicate that there is an increase in sp^2 character on going from the glycosyl-enzyme to the transition state of its hydrolysis, which would not be possible if the intermediate were ionic [10].

On the other hand, the nucleophilic character of the enzyme carboxylate involved in glycosyl-enzyme stabilization was first appreciated by reaction with conduritol epoxides and epoxyalkyl glycoside inhibitors [15–17]. Later on, the use of 2-deoxy-2-fluoro glycosides as mechanism-based inactivators [18] provided a valuable tool for the labelling and identification of the catalytic nucleophiles in a number of retaining glycosidases [19–23]. The fluorine at C-2 destabilizes the transition states for glycosyl-

Scheme 1 Mechanism of a retaining β -glycosidase by a double-displacement reaction through a covalent glycosyl-enzyme intermediate

Abbreviations used: MALDI-TOF-MS, matrix-assisted laser desorption ionization–time-of-flight-MS; G4G3G-2,4DNP, 2,4-dinitrophenyl 3-O- β -cellobiosyl- β -D-glucopyranoside; G4G3G-MU, 4-methylumbelliferyl 3-O- β -cellobiosyl- β -D-glucopyranoside; EAC, 1-(4-azonia-4,4-dimethylpentyl)-3-ethylcarbodi-imide iodide.

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enzyme formation and hydrolysis, thus slowing both steps, while the use of a reactive leaving group (such as fluoro or 2,4-dinitrophenyl aglycones) accelerates the glycosylation step sufficiently that the enzyme accumulates as its covalent glycosyl-enzyme adduct [24]. The trapped intermediate is catalytically competent, since addition of a glycosyl acceptor promotes turnover and generation of transglycosylation products. Not only the inductive effect of the fluorine at C-2, but also the removal of specific interactions of the 2-OH group with the protein, are responsible for the generation of the long-lived intermediate. 1-Fluoro glycosyl fluorides have been shown to be substrates rather than enzyme inactivators [25], indicating that the inductive effect of the fluorine atom (that will be even stronger in the 1-fluoro glycosyl-enzyme intermediate) is not sufficient to explain the inactivation by 2-deoxy-2-fluoroglycosides.

Specific interactions between the 2-OH group of the substrate and the enzyme are important for transition state stabilization with contributions in the range of 8–10 kcal · mol⁻¹ (1 cal = 4.184 J) [26,27], but the identity of the amino acid residues involved is not clearly established. In the case of the β -1,4-glucanase Cex from *C. fimi* [28], where the three-dimensional structure of the covalent glycosyl-enzyme intermediate using an inactive mutant has been visualized by X-ray crystallography, the side-chain carbonyl oxygen of the catalytic nucleophile has been proposed to play this role: the 2-OH group of the sugar has a close, low-barrier type, H-bond interaction with the carboxylate of the nucleophile in the covalent complex. Since this interaction may become optimized geometrically and electronically as the transition state is reached, it may be a widespread mechanism of transition-state stabilization in β -glucosyl hydrolysing enzymes.

Removal of the catalytic nucleophile (generally the carboxylate side-chain of aspartic acid or glutamic acid residues) by mutation to alanine or glycine gives inactive enzymes (approx. 10⁶-fold reduction in k_{cat}). As shown for a number of retaining glycosidases [β -glucosidase (*Agrobacterium faecalis*) [29], exo-1,4- β -glucanase (*C. fimi*) [30], 1,3-1,4- β -glucanase (*Bacillus licheniformis*) [31], and β -glucosidase (*Sulfolobus solfataricus*) [32]], addition of azide or formate as an exogenous nucleophile to the nucleophile-less mutants restores the enzyme activity: azide rescue gives glycosyl azide products, whereas formate re-activation yields the 'normal' hydrolysis products.

Focusing on the *Bacillus* 1,3-1,4- β -glucanase, we report in the present study the detection and characterization of a long-lived α -glycosyl formate adduct arising from chemical rescue of the nucleophile-less mutant E134A by sodium formate. Since formate resembles the excised carboxylate side chain of the nucleophile residue, the adduct detected represents a true glycosyl-enzyme intermediate mimic without disturbing the 2-OH substrate-enzyme interactions.

Bacillus 1,3-1,4- β -glucanases (1,3-1,4- β -D-glucan 4-glucanohydrolases, EC 3.2.1.73) are family 16 endo-glycosidases (retaining glycosidases) that catalyse the hydrolysis of mixed-linked glucans containing β -1,3 and β -1,4 glycosidic bonds, such as cereal β -glucans and lichenans. Work in our group is being directed towards understanding the molecular mechanisms of catalysis and specificity of *Bacillus* 1,3-1,4- β -glucanases by combining mutational and kinetic studies (for a review see [33]).

MATERIALS AND METHODS

Enzymes and reagents

Sodium formate, ¹³C-labelled sodium formate and calcium chloride were purchased from Sigma Chemical Co. Barley β -glucan was obtained from Megazyme (Bray, County Wicklow,

Ireland). 4-Methylumbelliferone and 2,4-dinitrophenol were purchased from Fluka, and they were recrystallized from glacial acetic acid (Sigma Chemical Co.). The substrates 2,4-dinitrophenyl 3-O- β -cellobiosyl- β -D-glucopyranoside (G4G3G-2,4DNP) [34] and 4-methylumbelliferyl 3-O- β -cellobiosyl- β -D-glucopyranoside (G4G3G-MU) [35,36] were synthesized as described previously. All buffers and solutions for kinetic experiments and reaction monitoring were filtered (0.45 μ m) prior to use. Recombinant wild-type and E134A mutant 1,3-1,4- β -glucanases from *B. licheniformis* expressed in *E. coli* were prepared as previously reported [31]. Purity was higher than 95% as judged by SDS/PAGE, according to the method of Laemmli [37]. Enzyme concentrations were determined by UV spectrophotometry using $\epsilon_{280} = 3.55 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [38].

Enzyme kinetics with G4G3G-2,4DNP and G4G3G-MU

All kinetics were performed by following changes in UV absorbance due to the release of 4-methylumbelliferone or 2,4-dinitrophenol using matched 1 cm pathlength cells in a Cary 4E spectrophotometer with a Peltier temperature control system that maintained the cells at 30 °C. Rates of the enzyme-catalysed reactions were determined by incubating the enzyme at substrate concentrations ranging between $0.25 \times K_m$ and $8.5 \times K_m$ in citrate/phosphate buffer (6.5 mM citric acid/87 mM Na₂HPO₄; pH 7.2), 0.1 mM CaCl₂ and 0–4.8 M sodium formate for 5–90 min in the thermostatted cell holder. Reactions were initiated by addition of substrate to a preincubated mixture of buffer, calcium chloride, enzyme and exogenous nucleophile, and the absorbance changes at 365 nm for G4G3G-MU (release of 4-methylumbelliferone; $\Delta\epsilon_{365} = 5136 \text{ M}^{-1} \cdot \text{cm}^{-1}$) or 425 nm for G4G3G-2,4DNP (release of 2,4-dinitrophenol; $\Delta\epsilon_{425} = 6134 \text{ M}^{-1} \cdot \text{cm}^{-1}$) were monitored. Values of $\Delta\epsilon$ were carefully determined under the assay conditions as reported previously [31,34].

For pH studies of the reaction E134A + G4G3G-2,4DNP in the presence of 2 M sodium formate, kinetics were performed as above but in 11 mM citrate/11 mM phosphate (pH 4.5–8.0), 0.1 mM CaCl₂ and a constant ionic strength of 2.2 M (using added KCl), at 30 °C. The pH-dependent values of $\Delta\epsilon_{425}$ were accurately determined under the same conditions. Kinetic pK_a values were determined from non-linear regression fitting of the data to eqn (1):

$$k_{\text{cat}} = \frac{(k_{\text{cat}})_{\text{lim}}}{1 + 10^{(pK_{a1} - \text{pH})} + 10^{(\text{pH} - pK_{a2})}} \quad (1)$$

where $(k_{\text{cat}})_{\text{lim}}$ is the limit value of k_{cat} .

NMR monitoring

¹H-NMR monitoring of enzymic reactions was performed following the procedure reported in Viladot et al. [31]. ¹H-NMR spectra were recorded on a Varian Gemini 300 spectrometer at 25 °C in ²H₂O at p²H 7.3 (adjusted with 1 M NaO²H/²H₂O) at intervals of 5–60 min. Enzyme, substrate and nucleophile concentrations were: 20–50 nM E134A, 10 mM substrate and 2 M sodium formate.

Matrix-assisted laser desorption ionization–time-of-flight-MS (MALDI–TOF–MS) monitoring

MALDI–TOF mass spectra were acquired on a Bruker (Bremen, Germany) BIFLEX spectrometer equipped with a pulsed nitrogen laser (337 nm), in the reflectron, positive ion mode, using 19 kV acceleration voltage and 20 kV reflector voltage. 2,5-

Hydroxybenzoic acid (Aldrich Chemical Co.) was used as the ionization matrix. Samples were prepared by diluting aliquots of the reaction mixture 100-fold in a 10 mg/ml solution of the matrix in water. From this solution, 1 μ l was spotted on to the sample slide and allowed to evaporate to dryness. Spectra obtained from 40–60 shots across the sample surface were accumulated for each sample.

HPLC monitoring of transglycosylation

Transglycosylation reactions between the 2,4-dinitrophenyl glycoside and 4-methylumbelliferyl β -cellobioside catalysed by the E134A mutant in the presence of sodium formate were performed in H₂O at 25 °C (pH 7.25). The reaction contained 12.0 μ M E134A, 10 M G4G3G-2,4DNP, 2 M formate and 40 mM G4G-MU. The initial volume of the reaction was 200 μ l. At different reaction times (0, 0.75, 1.5, 2, 3, 4, 5, 6, 7, 8.5, 10 and 24 h), samples of 4 μ l were added to 36 μ l of water and frozen at –20 °C, until HPLC and MALDI–TOF analyses were performed. Chromatographic (HPLC) conditions were as follows: Nova-pak[®] column C₁₈ (3.9 mm $\times$ 150 mm), 4 μ m particle size (Waters), 1 ml/min flow rate, 37 °C, methanol/H₂O was the eluent [18:82, (v/v)], and UV–visible detector (λ 316 nm).

RESULTS AND DISCUSSION

Chemical rescue by formate

Removal of the catalytic nucleophile Glu¹³⁴ in the *B. licheniformis* 1,3-1,4- β -glucanase by mutation to alanine (E134A mutant) was reported to largely inactivate the enzyme, with a 10⁶-fold reduction of k_{cat} with aryl β -glycoside substrates [31] (Table 1). Azide re-activated the mutant enzyme to yield a stable α -glycosyl azide product as the result of a single inverting displacement of the aglycone leaving group by azide when using the activated 2,4-dinitrophenyl β -glycoside (**1**). We also showed [31] that formate, which resembles the structure of the excised carboxylate side

chain of Glu¹³⁴ more closely, rescued the E134A mutant to give the hydrolysis products. Notably, a transient intermediate with a half-life of approx. 2 h was detected by ¹H-NMR before the final hydrolysis products were obtained (Scheme 2). It was tentatively assigned to the α -glycosyl formate adduct (**3**), which was then hydrolysed to give a mixture of the α and β -saccharides (**4 α**) and (**4 β**). Whether this transient intermediate was kinetically competent, and which was the stereochemistry of its initial hydrolysis product arising from either enzymic or spontaneous hydrolysis remained unclear.

¹H-NMR monitoring and stereochemical course

The reaction of E134A with substrate G4G3G-2,4DNP in the presence of 2 M sodium formate was monitored by ¹H-NMR at two different enzyme concentrations (11.4 and 23.6 μ M) in buffered ²H₂O (p²H 7.25) at 25 °C. The transient intermediate accumulated in sufficient concentration to be detected by ¹H-NMR (Figure 1). The new anomeric proton at δ 5.40 p.p.m. and J 7.5 Hz is consistent with an α -glycosyl formate adduct. It is stable enough to dissociate from the enzyme, since it accumulates up to a concentration of 2 mM for a reaction with 10 mM initial substrate and low enzyme concentration (11.4 μ M). The rate of formation and disappearance of the intermediate is enzyme-concentration dependent, proving that its hydrolysis is, at least partially, enzymic. The experiment at low enzyme concentration could not distinguish whether the first hydrolysis product from the transient intermediate was an α - or a β -glycoside, because the reaction was slower than mutarotation of the newly formed reducing end, so both anomers (α at δ 5.23 p.p.m. and J 3.5 Hz; β at δ 4.66 p.p.m. and J 8.1 Hz) were observed during the time course. At a higher enzyme concentration (23.6 μ M) (Figure 2), 28 % of the substrate was transformed after 15 min, from which 11 % accumulated as the α -glycosyl formate adduct and 17 % as the β -anomer of the hydrolysis product, no α -anomer being detected. As plotted in Figure 2, the concentration of the

Table 1 Kinetic parameters for wild-type and E134A mutant 1,3-1,4- β -glucanases in the absence and presence of exogenous formate

Parameters were measured in citrate/phosphate buffer (pH 7.2) containing 0.1 mM CaCl₂ at 30 °C. MU, 4-methylumbelliferyl; ND, not determined.

Enzyme	Substrate	k_{cat} (s ^{–1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (M ^{–1} ·s ^{–1})
Wild-type	G4G3G-MU (2)	0.67 $\pm$ 0.02	0.79 $\pm$ 0.04	850 $\pm$ 70
	G4G3G-2,4DNP (1)	603 $\pm$ 8	0.20 $\pm$ 0.01	(3.0 $\pm$ 0.2) $\times$ 10 ⁶
E134A	G4G3G-MU (2)	(4 $\pm$ 2) $\times$ 10 ^{–6}	ND	–
	G4G3G-2,4DNP (1)	(7.1 $\pm$ 0.1) $\times$ 10 ^{–4}	0.16 $\pm$ 0.02	4.4 $\pm$ 0.5
	with 2 M sodium formate	1.1 $\pm$ 0.2	0.4 $\pm$ 0.2	(2.7 $\pm$ 1.0) $\times$ 10 ³
	with 4 M sodium formate	2.1 $\pm$ 0.15	0.35 $\pm$ 0.04	(6 $\pm$ 1) $\times$ 10 ³

Scheme 2 Chemical rescue of the E134A 1,3-1,4- β -glucanase mutant by formate

Figure 1 ^1H -NMR monitoring of the reaction of G4G3G-2,4DNP with the 1,3-1,4- β -glucanase E134A mutant in the presence of sodium formate

The reaction was performed in $^2\text{H}_2\text{O}$ ($p^2\text{H}$ 7.25) at 25 °C. The reaction contained 23.6 μM E134A, 10 mM G4G3G-2,4DNP and 2 M formate. (a) Substrate at time 0 and (b–g) reaction after 15 min, 45 min, 1.5 h, 3.5 h, 7 h and 24 h. δ values in p.p.m. (J in Hz): G4G3G-2,4DNP (1), 4.48 (8.1) H-1C, 4.81 (8.1) H-1B, and 5.44 (7.5) H-1A; hydrolysis product, 4.49 (8.0) H-1C, 4.66 (8.1) H-1A (β -anomer), 4.76 (8.1) H-1B, and 5.23 (3.5) H-1A (α -anomer); α -glycosyl formate (* in the spectra), 5.40 (7.5) H-1A. For the substrate, 6' corresponds to H-6 of the 2,4-dinitrophenyl aglycone, and for the products, 6' corresponds to H-6 of 2,4-dinitrophenol.

α -anomer slowly increases to reach the final equilibrated α/β mixture (2:3) after a long reaction time.

Identification of the transient intermediate by MALDI-TOF-MS

To assess whether the exogenous formate was incorporated into the intermediate, the reaction was monitored by MALDI-TOF-MS. Figure 3(A) shows the recorded spectra for the time course of the reaction of E134A (12 μM) with 10 mM G4G3G-2,4DNP and 2 M sodium formate at pH 7.25. At zero time the expected peak of the 2,4-dinitrophenyl trisaccharide at m/z 693 [$M + \text{Na}$] $^+$ was not observed. Instead, fragmentation peaks arising from photochemical reactions of the chromogenic 2,4-dinitrophenyl aglycone due to the laser excitation appeared at m/z 509 and 541 (the same type of fragmentations, although not assigned, were also observed for other 2,4-dinitrophenyl glycoside controls). At increasing reaction time, a new peak at m/z 555 first increases and then decreases, whereas a peak at m/z 527, corresponding to

Figure 2 Time course of the ^1H -NMR integrals for the reaction of G4G3G-2,4DNP with the 1,3-1,4- β -glucanase E134A mutant in the presence of sodium formate

Relative integrals are taken from Figure 1 and expressed as molar ratios (in terms of percentage). (●) G4G3G-2,4DNP (1 in Scheme 2), (○) G4G3G- αOCHO (3 in Scheme 2; α -glycosyl formate), (▲) G4G3G- βOH (4 β in Scheme 2) and (△) G4G3G- αOH (4 α in Scheme 2).

the hydrolysis product, increases and it is the only final reaction product. The 555 m/z peak is assigned to the glycosyl formate intermediate. When the reaction was carried out with ^{13}C -labelled formate, the transient peak appeared at m/z 556 (Figure 3B), and when an equimolar mixture of sodium [^{13}C]formate and sodium [^{13}C]formate was used as exogenous nucleophile, two peaks of equal intensity at m/z 555 and 556 were observed. It was then proved that the exogenous formate was incorporated into the transient compound, and the coupling constant in the ^1H -NMR spectra indicates that it is the α -anomer.

Kinetics of E134A re-activation by formate

Figure 4 summarizes the kinetic parameters of the E134A mutant 1,3-1,4- β -glucanase as a function of formate concentration when monitoring the release of the 2,4-dinitrophenyl aglycone from G4G3G-2,4DNP by UV spectrophotometry. Reactions were performed in citrate/phosphate buffer (pH 7.2) at 30 °C, and initial velocities at different substrate concentrations were determined at < 3 % conversion. No saturation was obtained up to 5 M formate. At the highest formate concentration assayed, k_{cat} was increased 3000-fold relative to the formate-free reaction, and it was only 300-fold lower than the k_{cat} value for the wild-type enzyme with the same substrate (Table 1). Since K_m values do not change too much (≤ 2 -fold), the same large re-activation is seen for k_{cat}/K_m .

The pH dependence of k_{cat} at 2 M formate gave a bell-shaped curve (Figure 5) with two kinetic pK_a values of 5.5 and 6. These values reflect the structural features of the E134A mutant with bound formate when they are compared with those from the pH profile of the wild-type enzyme.

The hydrolytic reaction of aryl β -glycosides catalysed by the wild-type enzyme [39] shows kinetic pK_a values of 5.5 and 7.0 for k_{cat}/K_m (assignable to the free enzyme), whereas those for k_{cat} (assignable to the enzyme–substrate complex) are < 5 and 7.3–7.8

Figure 3 Formation and hydrolysis of the α -glycosyl formate monitored by MALDI-TOF-MS

Reaction of G4G3G-2,4DNP with the E134A 1,3-1,4- β -glucanase mutant and sodium formate was performed in H_2O (pH 7.25) at 25 °C. The reaction contained 12 μM E134A, 10 mM G4G3G-2,4DNP and 2 M formate. **(A)** Time course of the reaction. (a) Substrate at time 0 and (b–g) reaction after 1, 3, 9, 13, 24 and 48 h. m/z : 509 and 541 (G4G3G-2,4DNP, fragmentation peaks arising from photochemical reactions of the chromogenic 2,4-dinitrophenyl aglycone due to the laser excitation); 527 [G4G3G + Na] $^+$; 555 [G4G3G- αOCHO + Na] $^+$. **(B)** Spectra after reaction for 1.5 h (a) with 2 M $\text{H}^{12}\text{COONa}$, (b) with 1 M $\text{H}^{12}\text{COONa}$ + 1 M $\text{H}^{13}\text{COONa}$ and (c) with 2 M $\text{H}^{13}\text{COONa}$. m/z : 555, [G4G3G- $\alpha\text{O}^{12}\text{CHO}$ + Na] $^+$; 556, [G4G3G- $\alpha\text{O}^{13}\text{CHO}$ + Na] $^+$.

Figure 4 Kinetics of E134A re-activation by formate for the hydrolysis of G4G3G-2,4DNP

Conditions are given in Table 1.

(depending on the substrate). The $\text{p}K_a$ in the basic limb of the k_{cat}/K_m profile (7.0) was assigned to the general acid residue Glu¹³⁸ in the uncomplexed enzyme, since the same $\text{p}K_a$ value was also obtained from the pH-dependence of enzyme inactivation by the water-soluble carbodi-imide 1-(4-azonia-4,4-dimethylpentyl)-3-ethylcarbodi-imide iodide (EAC; inactivation due to chemical modification by EAC follows the ionization of an essential carboxylic acid residue that reacts in its protonated form). The $\text{p}K_a$ of the general acid/base residue of retaining glycosidases changes during the enzyme cycle. For the same residue to act as a general acid in the first step and act as a

Figure 5 pH-dependence of k_{cat} for the E134A 1,3-1,4- β -glucanase-catalysed reaction of G4G3G-2,4DNP in the presence of 2 M formate at 30 °C

Data were fitted to eqn (1). $\text{p}K_{a1} = 5.5$, $\text{p}K_{a2} = 6.0$ and $(k_{\text{cat}})_{\text{lim}} = 4.54 \text{ s}^{-1}$.

general base in the second step (Scheme 1), a significant shift in the $\text{p}K_a$ of the carboxy side chain must occur, as demonstrated by ^{13}C -NMR titration of a xylanase from *B. circulans* [40]. Upon formation of the glycosyl-enzyme intermediate, the $\text{p}K_a$ of the residue that acted as a general acid in the glycosylation step decreased approx. 2.5 pH units, due to the elimination of the negative charge of the nucleophile residue (now covalently linked to the ligand), being able to behave as a general base in

Scheme 3 Transglycosylation of the α -glycosyl formate (3) with 4-methylumbelliferyl β -cellobioside (5) catalysed by the E134A 1,3-1,4- β -glucanase mutant

the deglycosylation step. For the same reason, mutation of the nucleophile residue to an uncharged amino acid (glutamic acid to glutamine or alanine) results in a decrease of the pK_a assigned to the general acid [40].

For the E134A mutant in the presence of formate, the pK_a of 6.0 for k_{cat} arises from a downward shift of 1.8 pH units relative to the pK_a for k_{cat} of the wild-type enzyme with the same substrate, G4G3G-2,4DNP. Since formate is weakly bound to the active site and it mimics the excised glutamic acid of the nucleophile residue, the pK_a of the general acid (Glu¹³⁸) is not as low as expected for the nucleophile-less mutant.

On the other hand, the pK_a in the acidic limb of 5.5 for the E134A + formate reaction may reflect the ionization of the enzyme-bound formate anion in the enzyme–substrate complex. This value is similar to that determined in the wild-type enzyme (5.5 in the free enzyme, < 5 in the enzyme–substrate complex, tentatively assigned to the nucleophile Glu¹³⁴ [39]), consistent with the function of the formate anion playing the role of the excised catalytic nucleophile.

Formate rescue is very efficient, within two orders of magnitude of that of the wild-type, but requiring a large excess of the exogenous nucleophile. Since the catalytic reaction requires the formation of a ternary complex (E134A mutant–formate–substrate), and no saturation of k_{cat} on formate concentration was observed, productive binding of formate to act as a nucleophile is weak and still far from being as efficient as the carboxylate nucleophile in the active site of the wild-type enzyme, which is covalently attached to the enzyme. Moreover, less reactive substrates, such as the 4-methylumbelliferyl glycoside, G4G3G-MU, are not hydrolysed by the formate-assisted reaction, pointing to the importance of proper positioning and pre-organization of the reactants as critical components of enzyme catalysis [41].

Transglycosylation of the α -glycosyl formate

The hydrolysis of the α -glycosyl formate (3) is, at least partially, enzymic, because it is enzyme-concentration dependent. Compound 3 is a glycosyl-enzyme mimic in the β -glycosidase reaction, and as such it might act as a donor in the enzymic transglycosylation with a glycoside acceptor, thereby proving its kinetic competence in the overall process.

To a mixture of 2,4-dinitrophenyl glycoside (1) and E134A in the presence of 2 M formate, 4-methylumbelliferyl β -cellobioside

Figure 6 HPLC monitoring of transglycosylation of G4G3G-2,4DNP + 4-methylumbelliferyl β -cellobioside catalysed by E134A 1,3-1,4- β -glucanase in the presence of sodium formate

Chromatograms of (A) substrates at time 0, and (B) after reaction for 3 h. Retention times in min: 2,4-dinitrophenol, 1.57; G4G3G-2,4DNP (1 in Scheme 3), 5.67; 4-methylumbelliferyl β -cellobioside (5 in Scheme 3; glycosyl acceptor), 6.15; G4G3G4G4G-MU (6a in Scheme 3), 7.56; G4G3G4G4G3G4G4G-MU (6b in Scheme 3), 10.37; free 4-methylumbelliferone, 19.40.

(5) was added as glycosyl acceptor, and the reaction was monitored by HPLC (Scheme 3 and Figure 6). A peak corresponding to a chromogenic saccharide with a higher degree of polymerization was formed. MALDI-TOF-MS of the reaction mixture proved that different transglycosylation products were obtained: a major peak at m/z 527 $[M + Na]^+$ corresponds to the hydrolysis product, and a peak at m/z 1009 $[M + Na]^+$ is assigned to the 4-methylumbelliferyl pentasaccharide (6a). Also minor peaks at higher m/z ratios were detected: 1496 $[M + Na]^+$ (second transglycosylation product 6b), 1982 $[M + Na]^+$ (third transglycosylation product 6c), and 2468 $[M + Na]^+$ (fourth transglycosylation product 6d). These high-molecular-mass saccharides arise from the sequential condensation of the transglycosylation products with the trisaccharide donor as shown in Scheme 3. When the reaction mixture was treated with the

wild-type 1,3-1,4- β -glucanase, all high-molecular-mass oligosaccharides were hydrolysed to give **4**+**5** (by HPLC and MALDI-TOF-MS; results not shown), thus proving that only β -1,4 glycosidic bonds were produced in the transglycosylation reaction, in accordance with the substrate specificity of the enzyme [39].

Under the experimental conditions used in this experiment, the main transglycosylation product (**6a**) was formed in only 15% yield, because hydrolysis of the α -glycosyl formate (**3**) was the predominant reaction. In a similar reaction recently reported for a β -glucosidase from *S. solfataricus* [42], transglycosylation was obtained in higher yields for the nucleophile-less mutant (glutamic acid to glycine) in the presence of formate, an α -glycosyl formate intermediate being invoked as glycosyl donor, but not characterized or detected. For the 1,3-1,4- β -glucanase system, the formate adduct is long-lived and it is able to dissociate from the enzyme (as seen by $^1\text{H-NMR}$), so spontaneous hydrolysis in solution is also an important process that reduces the efficiency of the enzymic transglycosylation reaction.

The glycosynthase concept, the transglycosylation of an α -glycosyl fluoride donor with an acceptor catalysed by a mutant β -glucosidase lacking its catalytic nucleophile, was first introduced by S. G. Withers and co-workers on an exo-enzyme, the *A. faecalis* β -glucosidase [43], and later extended to endo-enzymes in our (on the 1,3-1,4- β -glucanase [44]) and other (on the cellulase Cel7A [45]) laboratories. The α -glycosyl fluoride acts as a glycosyl-enzyme mimic and reacts with the acceptor, but the newly formed product is not hydrolysed, because the mutant enzyme is hydrolytically inactive. The α -glycosyl formate reported in the present study can be seen as a donor formed *in situ* from a β -glycoside for the glycosynthase reaction. In this case, however, the reaction is not very efficient, due to the spontaneous hydrolysis of the formate adduct. With α -glycosyl fluorides instead, the E134A 1,3-1,4- β -glucanase is a highly efficient glycosynthase, with yields as high as 98% depending on the acceptor [44], the reaction being currently applied to the enzymic assembly of oligosaccharides.

Conclusions

The hydrolytic activity of the nucleophile-less E134A mutant 1,3-1,4- β -glucanase is restored by exogenous formate in a concentration-dependent manner. Few other retaining glycosidases mutated at the catalytic nucleophile have been reported to be rescued by formate [*A. faecalis* β -glucosidase [29], *C. fimi* exo- β -1,4-glycanase Cex [30], and *S. solfataricus* β -glycanase [32]], but, to the best of our knowledge, no transient intermediates have ever been detected. For the 1,3-1,4- β -glucanase system reported in the present study, a long-lived α -glycosyl formate adduct has been identified, proving that the exogenous nucleophile is able to play the role of the excised enzyme nucleophile. Since this transient intermediate is, at least partially, enzymically hydrolysed, and able to act as a donor in a transglycosylation (glycosynthase-type) reaction, it represents a true covalent glycosyl-enzyme intermediate mimic containing an unmodified sugar. This finding provides further evidence of the covalent nature of the glycosyl-enzyme intermediate in the reaction mechanism of retaining glycosidases, but, as opposed to the trapping experiments with 2-deoxy-2-fluoro glycosides, the 2-OH group of the substrate remains intact as in the natural substrate.

This work was supported by grants BIO97-0511-C02-02 (to A. P.) from the Comisi3n Interministerial de Ciencia y Tecnologa, Madrid, Spain, and 1999SGR0036 from the Generalitat de Catalunya.

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Received 10 November 2000/12 December 2000; accepted 17 January 2001