Presteady-state kinetics of *Bacillus* 1,3-1,4- β -glucanase: binding and hydrolysis of a 4-methylumbelliferyl trisaccharide substrate

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In the present study the first stopped-flow experiments performed on *Bacillus* 1,3-1,4- β -glucanases are reported. The presteady-state kinetics of the binding of 4-methylumbelliferyl 3-*O*- β -cellobiosyl- β -D-glucoside to the inactive mutant E134A, and the wild-type-catalysed hydrolysis of the same substrate, were studied by measuring changes in the fluorescence of bound substrate or 4-methylumbelliferone produced. The presteady-state traces all showed an initial lag phase followed by a fast monoexponential phase leading to equilibration (for binding to E134A) or to steady state product formation (for the wild-type reaction). The lag phase, with a rate constant of the order of 100 s⁻¹, was independent of the substrate concentration; apparently an induced-fit mechanism governs the formation of enzyme–

substrate complexes. The concentration dependencies of the observed rate constant of the second presteady-state phase were analysed according to a number of reaction models. For the reaction of the wild-type enzyme, it is shown that the fast product formation observed before steady state is not due to a rate-determining deglycosylation step. A model that can explain the observed results involves, in addition to the induced fit, a conformational change of the productive ES complex into a form that binds a second substrate molecule in a non-productive mode.

Key words: fluorescence, induced-fit, inhibition, mechanism, stopped-flow.

INTRODUCTION

The enzymic hydrolysis of glycosidic bonds in oligo- and polysaccharides, as well as in their glycoconjugates, is catalysed by a vast number of glycosyl hydrolases. These enzymes are currently classified into 85 families according to amino acid sequence similarities [1,2] (<http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html>), where each family member exhibits the same stereochemical outcome in the hydrolytic reaction: retention or inversion 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. For the last decade, mutational, mechanistic and structural studies have established many details of the mechanism of action of retaining glycosidases,

such as the identity of the catalytic residues (acid/base and nucleophile), the covalent nature of the glycosyl-enzyme intermediate, substrate distortion in the enzyme–substrate complex, etc. (for reviews see [3–5]).

Presteady-state kinetics is a valuable tool for describing individual steps in enzyme mechanisms [6]. Some interesting insights have been obtained using stopped-flow techniques for a number of glycosidases, such as β -glucosidases [7,8], glucosylases [9–14], xylanases [15,16] and cellulases [17], where slow conformational changes coupled to substrate binding have been detected in some cases [11].

In the framework of our structure–function studies of *Bacillus* 1,3-1,4- β -glucanases (for a review see [18]), we report in the present study the first stopped-flow experiments performed on these endo-acting glycosidases in order to obtain a better understanding of substrate binding and the hydrolysis mechanism.

Scheme 1 Double-displacement mechanism of a retaining glycosidase with formation of a covalent glycosyl-enzyme intermediate

Abbreviations used: G4G3G-MU, 4-methylumbelliferyl 3-*O*- β -cellobiosyl- β -D-glucoside; MU, 4-methylumbelliferone.

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Bacterial 1,3-1,4- β -glucanases (also known as 1,3-1,4- β -D-glucan 4-glucanohydrolases or lichenases; EC 3.2.1.73) are endo-depolymerases (family 16 of glycosyl hydrolases), which hydrolyse polysaccharides with mixed β -1,3 and β -1,4 glycosidic bonds, such as cereal β -glucans and lichenans, with a strict cleavage specificity for β -1,4 linkages in 3-*O*-substituted glucopyranosyl units [19–21]. The *Bacillus licheniformis* enzyme has been kinetically characterized under steady-state conditions using two families of chromogenic substrates, 4-methylumbelliferyl (Glc β p1,4)_nGlc β p1,3Glc β p [22] and Glc β p1,4Glc β p1,3Glc β p-aryl (where aryl corresponds to nitro-substituted phenyl aglycons) [23]. The hydrolysis proceeds with retention of the anomeric configuration [21], where the glycosylation step leading to the glycosyl-enzyme intermediate is rate determining at pH 7.2 for all aryl glycoside substrates assayed [23]. The catalytic residues, identified by mutagenesis and chemical rescue experiments [24,25], are Glu¹³⁸ as a general acid/base residue, and Glu¹³⁴ as a catalytic nucleophile. Two kinetic pK_a values of 5.5 and 7.0 were determined for the free enzyme (pH dependence of k_{cat}/K_m), the higher value assigned to the general acid residue based on chemical modification experiments with 1-ethyl-3-[3-(dimethylamino)-propyl]carbodi-imide [22].

The three-dimensional structure of the free enzyme at 0.18 nm resolution has been solved by X-ray crystallography [26], showing a β -sandwich jelly-roll architecture, which characterizes family 16 of the glycosyl hydrolases. Out of eight tryptophan residues three are present in the binding-site cleft, but only Trp²²¹ in subsite +I appears to have a stacking interaction with the substrate based on the modelled structure of an enzyme–substrate complex [27]. No changes in the intrinsic protein fluorescence have been observed upon binding of substrates and inhibitors under steady-state conditions. For this reason, the presteady-state studies reported in the present paper, using the stopped-flow technique, measure the changes in fluorescence of a fluorogenic 4-methylumbelliferyl 3-*O*- β -cellobiosyl- β -D-glucopyranoside (G4G3G-MU) substrate either upon binding or hydrolysis due to 4-methylumbelliferone (MU) production.

EXPERIMENTAL

Enzymes and substrate

The inactive E134A mutant of the 1,3-1,4- β -glucanase from *B. licheniformis* was obtained using PCR site-directed mutagenesis as previously reported [25]. Wild-type and E134A mutant 1,3-1,4- β -glucanases were expressed in *Escherichia coli* TG1 cells. The proteins were purified from the culture supernatant by modification of the previously reported protocol [25]; an ion-exchange chromatography step was substituted with a gel filtration step. The first step of the purification procedure involves cation-exchange chromatography on CM-Sepharose (Pharmacia Biotech Inc.) in 5 mM sodium acetate buffer (pH 5.5), where proteins are eluted with a linear gradient of 0–0.2 M NaCl in the same buffer. The second step is gel-filtration chromatography on Superose 75 (Pharmacia Biotech Inc.) using 20 mM CaCl₂/0.1 M NaCl/20 mM sodium acetate buffer (pH 6.0) at a flow rate of 0.3 ml·min⁻¹. After dialysis against 2 mM Mops/0.1 mM CaCl₂ (pH 7.0), and then against water, the proteins were freeze-dried for storage. Purity was higher than 95% as judged by SDS/PAGE according to the method described by Laemmli [28]. Protein concentration was determined spectrophotometrically at 280 nm using a molar absorption coefficient of 67 400 M⁻¹·cm⁻¹, based on the number of tyrosine and tryptophan residues in the protein [29].

Figure 1 Emission spectrum of MU

Under these conditions (1 μ M MU, pH 7.2, 30 °C, excitation = 360 nm) the G4G3G-MU substrate showed negligible emission (< 0.25 fluorescence arbitrary units at λ > 455 nm).

MU was obtained from Fluka, and was recrystallized from acetic acid. The substrate G4G3G-MU was synthesized as previously described [30].

Stopped-flow fluorescence experiments

General

The presteady-state kinetic experiments were performed at 30 °C in an Applied Photophysics DX17MV and/or a Hi-Tech Scientific PQ/SF-53 stopped-flow instrument each equipped with high-intensity xenon arc lamps, and thermostated syringe holders and reaction cuvettes. The excitation wavelength was either 350 nm with a slit width of 2 mm, where both the substrate and MU absorb, or 360 nm with a slit width of 5 mm, where only MU absorbs, as seen from fluorescence spectra of G4G3G-MU and of MU obtained at a number of excitation wavelengths and measured in a Perkin-Elmer λ -17 fluorimeter. Figure 1 shows an example. The light emitted from the reaction mixture passed cut-off emission filters (GG400 for the substrate measurements, and GG400 or WG455 for the MU measurements), so that an integrated fluorescence intensity was obtained. After rapid mixing (less than 1 ms) the time course of the fluorescence (arbitrary units expressed in V) was recorded taking 1000 or 400 pairs of data per experiment. Different sets of data (4–5) obtained using the same experimental conditions were measured and averaged. The resulting traces were then fitted to the appropriate non-linear analytical equations using the SX18MV v4.36 or the Hi-Tech software.

Substrate binding to the inactive E134A mutant

Experiments with the E134A 1,3-1,4- β -glucanase mutant were performed with G4G3G-MU concentrations ranging from 0.05 to 6.5 mM using 23 μ M enzyme at 30 °C in 87 mM Na₂HPO₄/6.5 mM citric acid (pH 7.2)/0.1 mM CaCl₂. These experiments all resulted in curves showing a single exponential decay after a lag phase. Eqn (1) gave the best fit to the experimental data not considering the lag-phase:

$$\Delta F([S], t) = \Delta F([S], \infty)[1 - \exp(-k_{obs} \cdot t)] \quad (1)$$

where k_{obs} is the observed first order rate constant, $\Delta F([S], t)$ is the relative fluorescence change observed at the actual substrate

concentration, $[S]$, and time, t , and $\Delta F([S], \infty)$ is the maximal fluorescence change obtained when equilibrium prevails. Values for k_{obs} and $\Delta F([S], \infty)$ at each substrate concentration were obtained from fits to eqn (1).

Stopped-flow experiments studying the intrinsic protein fluorescence (excitation at 280 nm and emission cut off filter WG320) of the wild-type 1,3-1,4- β -glucanase using 3-*O*- β -cellobiotrioseyl-D-glucose, a known competitive inhibitor [22], as the substrate, showed no changes in fluorescence (results not shown).

Kinetics of the wild-type enzyme

The presteady-state kinetics of the wild-type 1,3-1,4- β -glucanase catalysed hydrolysis of G4G3G-MU were studied using a series of substrate concentrations in the range 0.3–5 mM and approximately 10 μ M enzyme at 30 °C in 87 mM phosphate buffer/0.1 mM CaCl_2 , at three different pH values (6.9, 7.2 and 7.8), adjusted with the addition of citric acid. MU fluorescence (excitation wavelength of 360 nm) standard curves were determined under the same conditions, but in the absence of enzyme. MU solutions (0–20 μ M) were mixed in the stopped-flow instrument with buffer or with different substrate solutions (0–5 mM), and data from two different experiments were averaged. The MU fluorescence intensity coefficient, α_s , varies with the substrate concentration as follows:

$$\alpha_s = A \cdot \exp(-k \cdot [\text{G4G3G-MU}]) + B$$

where the pH-dependent values are: pH 6.9, $A = 7.25 \pm 0.6$, $k = 0.8 \pm 0.1$ and $B = 12.5 \pm 0.6$; pH 7.2, $A = 13 \pm 3$, $k = 0.5 \pm 0.3$ and $B = 9 \pm 3$; pH 7.8, $A = 28.8 \pm 3$, $k = 0.54 \pm 0.2$ and $B = 37.3 \pm 3$.

The fluorescence emission intensity of the substrate is approx. 1500 times smaller than that of MU. In all kinetic experiments a lag phase followed by a single exponential presteady-state phase and a constant steady-state production of fluorescence was observed. Eqn (2) showed the best fit to the experimental data not considering the lag phase:

$$\Delta F([S], t) = \Delta F_p [1 - \exp(-k_{\text{obs}} \cdot t)] + vt \quad (2)$$

where $\Delta F([S], t)$ is the relative fluorescence change observed at the actual substrate concentration, $[S]$, and time, t , ΔF_p is the presteady-state value of fluorescence change obtained from extrapolation of the linear steady-state progress to zero time, v is the steady-state rate and k_{obs} is the observed first order rate constant characterizing the presteady-state phase. Values for k_{obs} , ΔF_p and v were obtained from fits of eqn (2) to the averaged data sets, and expressed in concentration units using the corresponding MU-fluorescence standard curve data.

RESULTS

Presteady-state kinetics with wild-type enzyme

The presteady-state kinetics of *B. licheniformis* β -glucanase-catalysed hydrolysis of G4G3G-MU was investigated using the stopped-flow technique to measure the change in fluorescence due to MU formation as a function of the substrate concentration. Figure 2 shows a typical kinetic trace in which three different phases can be differentiated. The same pattern was obtained in all cases. First a lag phase with no fluorescence change, followed by a fast production of MU decreasing mono-exponentially according to eqn (2) to reach the constant steady-state rate, v . The lag phase lasts for 30 ms and shows no dependence on the concentration of the substrate. It is apparently not due to an experimental artefact, since the same pattern was observed using two different instruments. The calculated

Figure 2 Typical stopped-flow trace obtained after mixing G4G3G-MU with wild-type *B. licheniformis* 1,3-1,4- β -glucanase in which the increase in fluorescence, due to MU production as a consequence of substrate hydrolysis, is monitored

As shown in the Figure, the increase in fluorescence decreases.

presteady-state kinetic parameters obtained from fits of eqn (2) to the data at the different substrate concentrations are shown in Table 1. Series of experiments were also performed at pH 6.9 and 7.8 (Table 2). The results of an analysis of the steady-state rates, v , according to the usual β -glucanase substrate-inhibited Michaelis type of equation are summarized in Table 3. The steady-state results are equal to those obtained using normal manual mixing techniques [22,23]. In spite of previous findings from steady-state kinetics with a family of aryl β -glycosides [23], the immediate impression of the events in the presteady state as seen from inspection of the curves (Figure 2) is that the deglycosylation step of Scheme 1 may be rate determining. A closer look at the data, however, shows that this is not so.

The double-displacement mechanism shown in Scheme 1 corresponds with the less detailed Scheme 2:

Scheme 2

A retaining glycosidase operating according to Scheme 2 would show a so-called burst of the leaving group (MU), if, and only if, $k_{\text{cat}} = k_3 \ll k_2$, corresponding with slow deglycosylation and fast(er) glycosylation. For this minimal general mechanism the presteady-state kinetic parameters would be [31,32]:

$$k_{\text{obs}} = [k_2 / (1 + K_s/[S])] + k_3 \quad (3)$$

where $K_s = K_m(k_3 + k_2)/k_3$, so $K_s \gg K_m$; and for $[S] \cong K_m \ll K_s$, as used in the present paper:

$$k_{\text{obs}} \approx (k_{\text{cat}}/K_m)[S] + k_{\text{cat}} \quad (4)$$

The theoretical k_{obs} values calculated according to eqn (4) (Table 1) are clearly not in accordance with the experimental k_{obs}

Table 1 Presteady-state parameters of *B. licheniformis* 1,3-1,4- β -glucanase with the substrate G4G3G-MU at pH 7.2

Experimental conditions: 87 mM phosphate/6.5 mM citrate (pH 7.2)/0.1 mM CaCl_2 , 30 °C, $[E] = 11.9 \mu\text{M}$. Reactions were monitored by spectrofluorimetry. Experimental values for k_{obs} and ΔF_p correspond to the fit of the averaged traces to eqn (2). The ΔF_p values are expressed in mV and converted into μM MU released according to the proportionality value of MU fluorescence at each substrate concentration, as indicated in the Experimental section.

[S] (mM)	Experimental			Calculated*	
	k_{obs} (s^{-1})	ΔF_p (mV)	ΔF_p (μM)	ΔF_p (μM)	k_{obs} (s^{-1})
4.875	60 ± 4	7.2	0.71	9.79	2.9
3.25	58 ± 2	11.9	1.03	8.94	2.0
2.45	60 ± 3	8.5	0.66	8.21	1.6
1.625	62 ± 2	10.8	0.73	6.96	1.2
1.25	63 ± 3	10.8	0.68	6.07	1.0
0.625	59 ± 2	8.1	0.44	3.67	0.7
0.313	57 ± 3	6.4	0.32	1.76	0.5

* The k_{obs} and ΔF_p values according to model 1 were calculated using eqn (4) and eqn (5) respectively.

Table 2 Presteady-state parameters of *B. licheniformis* 1,3-1,4- β -glucanase with the substrate G4G3G-MU at different pH values

Experimental conditions: 87 mM phosphate buffer (pH adjusted using citric acid), 0.1 mM CaCl_2 , 30 °C, $[E] = 8.9 \mu\text{M}$. Reactions were monitored by spectrofluorimetry. The values of ΔF_p are expressed both in mV and in μM MU released, as indicated in Table 1.

pH 6.9				pH 7.8			
[S] (mM)	k_{obs} (s^{-1})	ΔF_p (mV)	ΔF_p (μM)	[S] (mM)	k_{obs} (s^{-1})	ΔF_p (mV)	ΔF_p (μM)
4.85	62 ± 2	18.46	0.68	4.7	69 ± 6	4.4	0.11
3.23	74 ± 3	16.15	1.24	3.13	67 ± 3	12.3	0.29
2.425	72 ± 7	9.16	0.68	2.35	91 ± 5	13.8	0.30
1.615	85 ± 4	8.3	0.58	1.565	81 ± 3	10.4	0.21
1.213	59 ± 2	8.9	0.59	1.175	87 ± 3	9.6	0.18
0.606	60 ± 3	7.2	0.43	0.588	73 ± 4	6.4	0.11
0.303	52 ± 3	6.4	0.35	0.294	60 ± 4	5.6	0.09

Table 3 Steady-state parameters of *B. licheniformis* 1,3-1,4- β -glucanase with G4G3G-MU at different pH values from stopped flow experiments

Experimental conditions: 87 mM phosphate buffer (pH adjusted using citric acid), 0.1 mM CaCl_2 , 30 °C. pH 6.9: $[E] = 8.9 \mu\text{M}$ and $[S] = 0.3\text{--}4.85 \text{ mM}$. pH 7.2: $[E] = 11.2 \mu\text{M}$ and $[S] = 0.3\text{--}4.88 \text{ mM}$. pH 7.8: $[E] = 8.9 \mu\text{M}$ and $[S] = 0.3\text{--}4.7 \text{ mM}$. After rapid mixing (less than 1 ms) of the wild-type enzyme and the substrate the reactions were monitored by measuring MU fluorescence.

pH	K_m (mM)	k_{cat} (s^{-1})	K_i (mM)	k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
7.2	0.5 ± 0.1	0.27 ± 0.03	—	0.5 ± 0.2
7.8	0.6 ± 0.1	0.12 ± 0.01	6.2 ± 1.7	0.2 ± 0.05
6.9	0.37 ± 0.08	0.26 ± 0.02	6.4 ± 1.7	0.7 ± 0.2

values. The same conclusion is reached, when we look at the ΔF_p parameter [31,32]:

$$\Delta F_p = \alpha_s [E_0] / [1 + (K_m/[S])]^2 \quad (5)$$

where the fluorescence intensity coefficient, α_s , is obtained from the standard curve of fluorescence versus MU concentration at the actual substrate concentration. The theoretical ΔF_p values are also given in Table 1. The observed fast presteady-state production of MU thus cannot be explained by Scheme 2 with $k_3 \ll k_2$, fast glycosylation and rate-determining deglycosylation. The results obtained at the three pH values, where the catalytic acidic residue Glu¹³⁸ ($\text{p}K_a = 7.2$, determined from the pH de-

pendence of k_{cat} values) markedly changes its state of protonation, show only minor differences (Tables 1 and 2). If k_{obs} is mainly determined by the rate constant of the glycosylation step (k_2), a decrease in k_{obs} with increasing pH in this range would be expected.

Kinetics of binding to the inactive E134A mutant

To determine if the above presteady-state results could possibly reflect changes in the substrate fluorescence when binding occurs, in spite of the lack of signal from free substrate when the excitation wavelength is 360 nm and emission is obtained at

Figure 3 Typical stopped-flow trace obtained after mixing G4G3G-MU with the inactive mutant E134A of *B. licheniformis* 1,3-1,4- β -glucanase

The decrease in substrate fluorescence on binding was monitored.

Figure 4 k_{obs} dependence of substrate binding to the inactive mutant E134A on substrate concentration

Conditions: 87 mM Na_2HPO_4 /6.5 mM citric acid (pH 7.2)/0.1 mM CaCl_2 , 30 °C, 23 μM enzyme and different substrate concentrations ranging from 0.05 to 6.4 mM. Experiments were performed in triplicate. Results are presented as means $\pm$ S.D. Inset: magnification of the plot at low substrate concentrations.

wavelengths greater than 450 nm, series of similar experiments were performed using the inactive β -glucanase mutant E134A [25], but no change in fluorescence intensity was observed in these experiments. These results also exclude any significant contamination of the mutant preparation with the wild-type enzyme.

Binding of the substrate to the inactive mutant, E134A, did, however, occur, as seen from series of experiments using an excitation wavelength of 350 nm and obtaining emission at wavelengths greater than 400 nm, where both the substrate, G4G3G-MU, and the product, MU, absorb and give rise to fluorescence. Since MU is not formed by the inactive mutant, the

Figure 5 Maximal fluorescence change at equilibrium with inactive mutant E134A–G4G3G-MU complexes

(a) Dependence of $\Delta F([S], \infty)$ on substrate concentration. Conditions were the same as those described in the legend to Figure 4. (b) Hill plot based on the equilibrium values of the change in substrate fluorescence. The fractional saturation (y) is $\Delta F([S], \infty)/\Delta F([S], \infty)_{\text{max}}$. Hill coefficient $\cong 1.2$.

observed fluorescence is due to the substrate. In the present study the pattern in all cases was the same and similar to that of the presteady-state phase of the wild-type enzyme–substrate reaction. After a lag phase, where no change of signal was observed for approximately 30 ms irrespective of the substrate concentration (Figure 3), a monoexponential change in the fluorescence intensity according to eqn (1) was observed until a decreased equilibrium value was reached. Figures 4 and 5 illustrate the results. Interestingly, k_{obs} decreases as a function of the substrate concentration to reach a plateau at high concentrations, and the Hill plot corresponding to the signal change has a slope > 1 (Figure 5b).

DISCUSSION

The presteady-state kinetics of the reactions of the substrate G4G3G-MU with wild-type and inactive mutant E134A 1,3-1,4- β -glucanase from *Bacillus* were investigated. Scheme 1, which depicts the current understanding of the reaction mechanism of 1,3-1,4- β -glucanase, cannot explain the results obtained in the present study. According to Scheme 1 a fast presteady-state production of MU should be detected only if deglycosylation is rate determining and an enzyme-intermediate accumulates, and this is shown to be inconsistent with the quantities measured (Tables 1 and 2). Furthermore, Scheme 1 does not account for the known substrate inhibition [22], which implies that the enzyme can accommodate two oligosaccharide substrate molecules at the same time, in a manner that leads to decreased overall rates.

A lag phase lasting approx. 30 ms (that is independent of the substrate concentration) was observed in the production of MU by the wild-type enzyme, as well as in substrate binding by the inactive E134A mutant. This may be explained by a reaction model with two different conformations of the free enzyme, E' and E , where only E is able to bind the substrate and the equilibrium is shifted towards E' . A conformational change of E'

to E before the substrate can bind (an induced fit) is required, as in Scheme 3:

Scheme 3

If the lag-period is taken to be approx. five times the half-time of the conformational change a value of $k_f \cong 100 \text{ s}^{-1}$ is obtained.

The second presteady-state phase shows a decrease in k_{obs} as a function of substrate concentration (mM range) when the substrate binding is measured. Furthermore, binding of more than one substrate molecule is indicated. As seen in Figure 5(b) the slope of the Hill plot is greater than 1. On the other hand, a k_{obs} value by-and-large independent of the substrate concentration (mM range) is seen when product formation is measured. After having considered a number of more simple reaction models [10,11,33], and evaluated equations for an immediately possible one (see the Appendix), we had to discard these. However, we have found that Scheme 4, as a working model for substrate binding, can explain the results [34]: Scheme 4 of substrate binding to the inactive mutant:

Scheme 4

Scheme 5 of substrate binding to the active enzyme and product formation:

Scheme 5

After the relatively slow conformational change that accounts for the lag phase (k_f of the order of 100 s^{-1}), the second presteady-state phase is caused by a first substrate binding step that is followed by a conformational change and the binding of a second substrate molecule. ES is assumed to be the only productive enzyme-substrate complex and the rate of product formation is $v = k_{+2}[\text{ES}] = d[\text{MU}]/dt$.

Scheme 4: binding

The enzyme-substrate complexes ES^* and ES_2^* give rise to the signal observed in the substrate binding studies, whereas ES is

silent. The k_{obs} expression for the second phase in Scheme 4 is evaluated in [34], and results in eqn (6):

$$k_{\text{obs}} = \frac{k_{+3}}{1 + K_1/[S]} + \frac{k_{-3}}{1 + [S]/K_4} \quad (6)$$

This expression of k_{obs} of the second phase is a decreasing function of substrate concentration if $[S] \gg K_1$ and $[S] \cong K_4$ (mM range) and accounts for the experimental results obtained with the mutant. With $k_{+3} \cong 15 \text{ s}^{-1}$, $k_{-3} \cong 175 \text{ s}^{-1}$, $K_1 \cong 0.01 \text{ mM}$ and $K_4 \cong 0.05 \text{ mM}$ we get the curve shown in Figure 4. The same conditions also explain the lack of dependence of the lag phase on substrate concentration, since:

$$(k_{\text{obs}})_{\text{lag}} = k_r/(1 + [S]/K_1) + k_f \cong k_f \quad (7)$$

Scheme 5: product formation

For the active enzyme the steady-state kinetic rate corresponding to Scheme 5, assuming rate-determining glycosylation in agreement with previous steady-state results [23] is:

$$v = k_c[\text{E}_0]/(1 + K_m/[S] + [S]/K_i) \quad (8)$$

where $K_m = K_1(1 + K_0)/(1 + K_3)$, with the steady-state relation $[\text{ES}^*]/[\text{ES}] = K_3 = k_{+3}/k_{-3}$ ($K_0 = [\text{E}]/[\text{E}] = k_r/k_f$ and $K_1 = (k_{-1} + k_{+2})/k_{+1}$), $K_i = K_4(1 + K_3)/K_3$ and $k_c = k_{+2}/[1 + K_3]$.

k_{obs} is governed by the formation of ES^* and ES_2^* , so that product formation, $k_{+2}[\text{ES}]$, decreases from an initial value ($[\text{ES}^*] + [\text{ES}_2^*] = 0$) to the final steady-state value ($[\text{ES}^*] + [\text{ES}_2^*] > 0$), and therefore eqn (6) applies. According to eqn (6), the conditions for obtaining a (by-and-large) substrate-independent k_{obs} of the second presteady-state phase are $[S] \gg K_1$ and $[S] \ll K_4$ or $[S] \gg K_4$. We have used $[S] \cong K_m$ (actually 0.5–10-fold K_m for $[S] = 0.3$ –5 mM), if K_0 is large as is the case if the $[\text{E}]/[\text{E}]$ equilibrium is shifted towards E' , as we have suggested, and if the K_3 value is of the same order for the active enzyme as that estimated for the inactive mutant, $K_3 \cong 0.1$, or at least not much larger, we get $K_m \gg K_1$, which means that the first condition is valid. A small decrease in the K_4 value of the active enzyme compared with that of the mutant is sufficient to fulfil the other condition. Furthermore, this model may explain the small or absent k_{obs} -dependence on pH in the range 6.9–7.8, where according to steady-state experiments on the pH dependence of the wild-type 1,3-1,4- β -glucanase activity with G4G3G-MU, the $\text{p}K_a$ of the general acid catalyst, Glu¹³⁸, in the $\text{E} \cdot \text{S}$ complex is 7.2 [22]. Since k_{obs} is independent of k_{+2} [eqn (6)] deprotonation of Glu¹³⁸ may have no effect.

We have shown that the presteady-state kinetics of the 1,3-1,4- β -glucanase may be explained by Scheme 4 with two substrate binding steps and two enzymic conformational changes. The results definitely cannot be explained in terms of a slow step after glycosylation and release of the leaving group in the reaction pathway (Scheme 2 and the Appendix). Scheme 4 is rather complicated, but since apparently the hydrolytic apparatus of the retaining glucosidases and the actual bond-breaking mechanism (Scheme 1) is the same for a large number of individual enzymes, it seems highly likely that regulatory mechanisms like those invoked in Scheme 4 exist. Indeed, a related kinetic scheme has been observed on a β -glucosidase from plant cell-walls, where transient kinetics were explained by an hysteresis mechanism involving two interconvertible conformational forms of the enzyme [35].

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APPENDIX

If we consider that 1,3-1,4- β -glucanase operates by a double-displacement mechanism in which the MU release is rate determining, an extension of Scheme 2 may explain the observed presteady-state kinetics of the wild-type enzyme. A tentative model is:

Scheme A1

A slow MU dissociation step is included after a fast bond-breaking step in which MU is produced, so that the complex $E-P \cdot MU$ is accumulated. MU may be involved in a stacking interaction with Trp²²¹ which, according to a molecular model of a 1,3-1,4- β -glucanase-hexasaccharide complex [27], is positioned in the +I subsite. This leads to an initial fast production of fluorescence signal, ΔF_p , proportional to the accumulated $E-P \cdot MU$. The rate would be:

$$d([E-P \cdot MU])_t/dt = k_f[ES] - (k_2 + k_r)[E-P \cdot MU]$$

where the total amount of enzyme is:

$$[E_0] = [E] + [ES] + [E-P \cdot MU] + [E-P]$$

If the substrate binding step is considered to be in equilibrium, and $k_3 \gg k_2$:

$$[E_0] = (1 + K_1/[S])[ES] + [E-P \cdot MU]$$

so that:

$$d([E-P \cdot MU])_t/dt = k_f[E_0]/(1 + K_1/[S]) - (k_r + k_2 + k_f/(1 + K_1/[S]))[E-P \cdot MU]$$

and

$$[E-P \cdot MU]_{(t)} = (k_f[E_0]/((1 + K_1/[S])k_{obs}))(1 - \exp(-k_{obs}t)) \quad (A1)$$

where:

$$k_{obs} = k_r + k_2 + k_f/(1 + K_1/[S]) \quad (A2)$$

At steady state, after rearrangement of eqn (A1) we get:

$$\Delta F_p = \alpha'[E-P \cdot MU] = \alpha'[E_0]/[(1 + K)(1 + K_m/[S])] \quad (A3)$$

where:

$$K_m = K_1K/(1 + K), \text{ and} \quad (A4)$$

$$K = (k_r + k_2)/k_f \quad (A5)$$

and the proportionality coefficient, α' , may be different from that determined experimentally for MU, α , since the two fluorescent species, MU and $E-P \cdot MU$, may not have exactly the same fluorescence intensity.

A k_{obs} value independent of the substrate concentration is obtained only if $[S] \gg K_1$, but $[S]$ is of the same order as K_m and eqn (A4) requires $K_m < K_1$, so this is not possible. Moreover, no

dependence of the k_{obs} values on pH, as expected from the pH-dependency of the glycosylation step, is observed. We find that the model has to be discarded.

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