Pre-steady-state kinetics of *Bacillus licheniformis* 1,3-1,4- β -glucanase: evidence for a regulatory binding site

Mireia ABEL^{*†}, Karin IVERSEN^{*}, Antoni PLANAS[†] and Ulla CHRISTENSEN^{*1}

^{*}Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark, and [†]Laboratory of Biochemistry, Institut Químic de Sarrià, Universitat Ramon Llull, Via Augusta 390, E-08017 Barcelona, Spain

In a previous paper, we reported the first stopped-flow experiments on a *Bacillus licheniformis* 1,3-1,4- β -glucanase [Abel, Planas and Christensen (2001) *Biochem. J.* **357**, 195–202]. It was shown that the pre-steady-state kinetics of the 1,3-1,4- β -glucanase using the substrate 4-methylumbelliferyl 3-*O*- β -cellobiosyl- β -D-glucoside may be explained by a reaction scheme involving an induced fit and the binding of two substrates as well as a second enzymic conformational change, whereas the results definitely could not be explained in terms of the simple double-displacement scheme. In the present study, we report further stopped-flow kinetic results on the glucanase using a series of low-molecular-mass substrates with various leaving groups and varying chain length.

The analysis of the resulting data leads to the conclusion that the free enzyme exists in two conformations, one of which binds the substrates rather strongly in a regulatory site, before any productive interactions can take place. This corresponds to an allosteric activation mechanism. With these substrates, however, the productive enzyme–substrate species are also able to change into less active or inactive forms. This may be seen as a feedback inhibitory mechanism.

Key words: allosteric activation, reaction mechanism, stopped-flow kinetics.

INTRODUCTION

Glycosidases catalyse the hydrolysis of glycosidic bonds in oligo- and polysaccharides, as well as in their glycoconjugates. A large number of glycosidases (or glycosyl hydrolases; EC 3.2.1) have been identified in nature, and are currently classified in 87 families according to amino-acid-sequence similarities [1,2]. Members of each family exhibit the same stereochemical outcome in the hydrolytic reaction: inversion or retention of the anomeric configuration. The mechanism of bond cleavage of retaining glycosidases is a double-displacement reaction (as shown in Scheme 1 in [3], corresponding to the less detailed Scheme 2 [3]).

A glycosyl-enzyme intermediate is formed (glycosylation step) and hydrolysed (deglycosylation step) through oxo-carbenium ion-like transition states catalysed by two essential active site carboxyl groups that provide general acid/base and nucleophilic catalysis. Many details of the mechanism of action of these enzymes are known (reviewed in [3a–6]). A few glycosidases such as β -glucosidases [7,8], glucoamylases [9–13], xylanases [14,15] and cellulases [16] have been studied using the stopped-flow technique [17] to obtain mechanistic details from the pre-steady-state. Such studies have revealed conformational changes coupled to substrate binding as well as the existence of two or more active enzyme conformations, proving that more complex kinetic mechanisms differentiate individual enzymes beyond the common basic mechanism.

Bacillus licheniformis 1,3-1,4- β -glucanases (1,3-1,4- β -D-glucan 4-glucanohydrolases or lichenases; EC 3.2.1.73) have been thoroughly studied (reviewed in [18,19]). These enzymes are endo-depolymerases (family 16 glycosyl hydrolases), which hydrolyse polysaccharides with mixed β -1,3 and β -1,4 glycosidic bonds, such as cereal β -glucans and lichenins, with a strict cleavage specificity for β -1,4-glycosidic bonds in 3-*O*-substituted glucopyranosyl units [20,21]. The binding site in the three-dimensional jelly-roll β -sandwich structure [22,23] is an open cleft composed of six subsites (–4 to +2).

The steady-state kinetics of the *B. licheniformis* enzyme with two families of low-molecular-mass chromogenic substrates, (Glc β 4)_nGlc β 3Glc-MU (where MU is 4-methylumbelliferyl) [24] and Glc β 4Glc β 3Glc-aryl (where aryl represents nitro-substituted phenyl aglycones) [25] have been thoroughly investigated. Hydrolysis proceeds with retention of the anomeric configuration [21], and the glycosylation step leading to the glycosyl-enzyme intermediate is rate-determining at pH 7.2 for all aryl glycoside substrates assayed [5].

In a previous study, we reported the first stopped-flow experiments on a *B. licheniformis* 1,3-1,4- β -glucanase [3]. It was shown that the pre-steady-state kinetics of the 1,3-1,4- β -glucanase using the substrate 4-methylumbelliferyl 3-*O*- β -cellobiosyl- β -D-glucoside, may be explained with two substrate binding steps and an induced fit, as well as one more enzymic conformational change (Scheme 3 in [3]). The results definitely could not be explained in terms of Scheme 1 in [3].

To obtain a better understanding of the reaction mechanism, in the present paper, we report the stopped-flow kinetics of wild-type 1,3-1,4- β -glucanase from *B. licheniformis* using a series of low-molecular-mass substrates with various leaving groups and varying chain length. In the analysis of the data, we have found that the previously proposed scheme (Scheme 3 in [3]) is too simple to explain the results. We therefore present a new scheme that suggests the binding of a substrate molecule in a regulatory site and the occurrence of conformational changes of both the substrate molecule and its productive enzyme–substrate complex.

EXPERIMENTAL

Enzymes and substrates

1,3-1,4- β -glucanase from *B. licheniformis* was obtained as described previously in [27–29]. The 1,3-1,4- β -glucanase was expressed in *Escherichia coli* TG1 cells, and purified from

Abbreviations used: DNP, dinitrophenol; MU, 4-methylumbelliferyl.

¹ To whom correspondence should be addressed (e-mail ulla@fk.ki.ku.dk).

	Compound	n	X
1	G3G-MU	1	
2	G4G3G-MU	2	
3	G4G4G3G-MU	3	
4	G4G4G4G3G-MU	4	
5	G4G3G-3,4-DNP	2	
6	G4G3G-2,4-DNP	2	

Figure 1 Substrates used in the pre-steady-state kinetics of the 1,3-1,4- β -glucanase from *Bacillus licheniformis*

the supernatant through two chromatographic steps (cationic exchange and gel filtration). Enzyme purities were estimated to be higher than 95 % as determined by SDS/PAGE, according to Laemmli [30]. The protein concentration was determined spectrophotometrically at 280 nm using a molar absorption coefficient of $67\,400\text{ M}^{-1}\cdot\text{cm}^{-1}$, calculated on the basis of the number of tyrosine and tryptophan residues [31].

MU, 3,4-dinitrophenol (3,4-DNP) and 2,4-dinitrophenol (2,4-DNP) were obtained from Fluka, and were re-crystallized from acetic acid. The different substrates (Figure 1) were synthesized as previously reported: MU β -laminaribioside (1), MU 3-*O*- β -cellobiosyl- β -D-glucoside (2), MU 3-*O*- β -cellotriosyl- β -D-glucoside (3) and MU 3-*O*- β -cellotetraosyl- β -D-glucoside (4) as in [32], 3,4-DNP 3-*O*- β -cellobiosyl- β -D-glucoside (5) and 2,4-DNP 3-*O*- β -cellobiosyl- β -D-glucoside (6) as in [25].

Stopped-flow kinetics

The pre-steady-state and steady-state rates were measured in a Hi-Tech Scientific PQ/SF-53 stopped-flow instrument equipped with a high-intensity xenon arc lamp, thermostatically controlled syringe holder and reaction cuvette. MU-substrate experiments were performed monitoring the change of fluorescence (arbitrary units expressed in V) due to MU release. The excitation wavelength was 360 nm with a slide width of 5 mm, and the light emitted from the reaction mixture passed a WG455 cut-off emission filter so that an integrated fluorescence-intensity signal was obtained. The conversion of voltage into concentration was as previously described in [3]. For the DNP-substrates, the reactions were followed by measuring the change of absorbance at 400 nm due to the release of each DNP. After rapid mixing (less than 1 ms) the time course of the reactions were recorded taking

400 pairs of data per experiment. A mean was taken for each series of data ($n = 4-5$) obtained under the same experimental conditions. The resulting traces were then fitted to the appropriate non-linear analytical equations using the Hi-Tech software.

Experiments were performed at 30 °C in 6.5 mM citrate, 87 mM Na_2HPO_4 buffer, 0.1 mM CaCl_2 , pH 7.2, I 0.15. Enzyme and substrate concentrations are indicated in the legends to the Figures and Tables. The concentration of enzyme was chosen so that measurable signal changes were obtained, and the range of substrate concentrations was around their steady-state K_m values. All traces showed an initial lag phase followed by a single exponential pre-steady-state phase followed by a linear steady-state aglycone release. The equation that best fitted to the mean traces, not considering the lag phase was:

$$\Delta\text{Signal}(S, t) = \Delta\text{Signal}_p[1 - \exp(-k_{\text{obs}} \cdot t)] + v_{\text{ss}}t \quad (1)$$

where $\Delta\text{Signal}(S, t)$ is the relative change of signal observed at the actual substrate concentration, S , and time, t , ΔSignal_p is the value of the pre-steady-state change of signal obtained from extrapolation of the steady-state rate to zero time, v_{ss} is the initial steady-state rate and k_{obs} is the observed first-order rate constant that describes the pre-steady-state phase. The ΔSignal_p is converted to ΔP (molar concentration) as previously described in the experiments with MU-substrates (1, 2, 3 and 4) in which a change of fluorescence is monitored [27], and to ΔP (molar concentration using molar absorptance coefficients (at pH 7.2) of $6985\text{ M}^{-1} \cdot \text{cm}^{-1}$ for 2,4-DNP and $7500\text{ M}^{-1} \cdot \text{cm}^{-1}$ for 3,4-DNP in the experiments with the DNP-substrates (5 and 6) in which a change of absorbance is measured.

The v_{ss} values obtained from the fit of eqn (1) to the stopped-flow curves were fitted to the Michaelis-Menten equation or,

Figure 2 Typical stopped-flow trace obtained after mixing the substrate G4G3G-3,4-DNP (**5**) with the wild-type 1,3-1,4- β -glucanase

The increase of absorbance, due to 3,4-DNP production, is monitored over time.

when appropriate because of apparent substrate inhibition, to the following equation [24]:

$$v_{ss} = k_{cat} \cdot E_0 / (1 + K_m/S + S/K_i) \quad (2)$$

Eqn (2) includes the effect of substrate inhibition with inhibition constant K_i .

RESULTS AND DISCUSSION

The stopped-flow kinetics of wild-type 1,3-1,4- β -glucanase from *B. licheniformis* has been investigated using a series of low-molecular-mass substrates with different chain length (compounds **1–4**) and leaving groups (compounds **2**, **5** and **6**) (Figure 1). From thorough investigations of the steady-state kinetics of the *B. licheniformis* enzyme with two families of low-molecular-mass chromogenic substrates, (Glc β 4)_nGlc β 3Glc-MU [25] and Glc β 4Glc β 3Glc-aryl [3], it is documented that glycosylation is the rate-determining step of the hydrolysis and that the electron-withdrawing nature of the aglycone determines the rate. Thus the lower the pK, the faster the reaction, e.g. the k_{cat} of 2,4-DNP-substrates are approx. two orders of magnitude larger than the k_{cat} of MU-substrates. The specificity of the enzyme is primarily determined by subsites –3 and –2, thus a disaccharide as G3G-MU (**1**) is a very poor substrate [33]. In agreement with this, the G3G-MU (**1**) showed no change of signal in the stopped-flow experiments.

All other kinetic traces showed the same general behaviour, an initial lag phase followed by fast pre-steady-state aglycone production decreasing in a single exponential manner to a final slower steady-state release. A typical trace is shown in Figure 2. From fits of eqn (1) to the mean traces, not including the lag phase, series of values of the pre-steady-state parameters k_{obs} and ΔP , and the initial steady-state rates, v_{ss} , were obtained. Results are illustrated in Tables 1 and 2.

Steady-state kinetics

Steady-state kinetic parameter values obtained from fits of the usual substrate-inhibited Michaelis–Menten type of equation [Eqn (2)] to the v_{ss} data are shown in Table 3. As illustrated

Table 1 Example series of kinetic parameters calculated from fits of eqn (1) to 3–5 mean stopped-flow traces (30 °C, pH 7.2, / 0.15)

(a) [G4G3G-MU] (mM)	k_{obs} (s ^{–1})	$\Delta P/E_0$	v_{ss}/E_0 (s ^{–1})
4.88	60 ± 4	0.059	0.24
3.25	58 ± 2	0.087	0.27
2.45	60 ± 3	0.055	0.27
1.63	62 ± 2	0.061	0.26
1.25	63 ± 3	0.057	0.23
0.63	59 ± 2	0.037	0.18
0.31	57 ± 3	0.027	0.11
$E_0 = 11.9 \mu\text{M}$			
(b) [G4G3G-3,4-DNP] (mM)	k_{obs} (s ^{–1})	$\Delta P/E_0$	v_{ss}/E_0 (s ^{–1})
0.85	67 ± 2	0.36	2.8
0.56	64 ± 2	0.34	2.9
0.42	65 ± 2	0.30	2.4
0.21	55 ± 2	0.25	1.8
0.11	47 ± 2	0.14	0.92
0.05	50 ± 3	0.072	0.47
0.03	32 ± 6	0.037	0.23
$E_0 = 12.3 \mu\text{M}$			
(c) [G4G3G-2,4-DNP] (mM)	k_{obs} (s ^{–1})	$\Delta P/E_0$	v_{ss}/E_0 (s ^{–1})
2.40	39 ± 2	33	212
1.60	41 ± 2	28	218
1.20	39 ± 3	20	202
0.60	29 ± 3	10	180
0.30	33 ± 4	5.1	122
0.15	39 ± 4	1.6	64
0.04	37 ± 4	0.7	11.4
$E_0 = 0.98 \mu\text{M}$			

in Figure 3, the assessment of the individual values of K_m , k_{cat} , and K_i is difficult. The two curves shown in Figure 3 each represent reasonable fits to the data, but with significantly different parameter values; however, the value of k_{cat}/K_m is reliable. The sets of values listed in Table 3 are those of which $K_m < K_i$, in agreement with previous steady-state kinetic studies [24].

Pre-steady-state kinetics

The reactions initially show a lag phase, which lasts approx. 30 ms. Since the length of the lag phase is apparently independent of the substrate nature and concentration, an induced fit involving an $E \rightleftharpoons E^*$ step, which is slow compared with the formation of the fast product-producing enzyme–substrate complexes, is suggested. If we assume that the apparent length of time of the lag phase in all experiments, ≈ 0.03 s, is the time at which the initial reaction has run 95–99 % to completion, we can conclude that $t = 0.03$ s is roughly equal to 5 times the half-time, $t_{1/2}$ (corresponding to 97 % reaction), and that the apparent rate constant of the lag phase is of the order of magnitude ≈ 100 s^{–1}.

As observed previously for the G4G3G-MU substrate (**2**) [3], the k_{obs} values (Tables 1 and 2) that govern a change from fast to slow product formation are practically independent of substrate concentration in the K_m range, and show a tendency to decrease only at concentrations where the steady state approximation ($E_0 \leq 5$ –6 times the substrate concentration) becomes invalid. k_{obs} values are of the same order of magnitude for all substrate

Table 2 Example series of kinetic parameters calculated from fits of eqn (1) to 3–5 mean stopped-flow traces (30 °C, pH 7.2, / 0.15)

(a)			
[G4G3G-MU] (mM)	k_{obs} (s ⁻¹)	$\Delta P/E_0$	v_{ss}/E_0 (s ⁻¹)
4.88	60 ± 4	0.059	0.24
3.25	58 ± 2	0.087	0.27
2.45	60 ± 3	0.055	0.27
1.63	62 ± 2	0.061	0.26
1.25	63 ± 3	0.057	0.23
0.63	59 ± 2	0.037	0.18
0.31	57 ± 3	0.027	0.11
$E_0 = 11.9 \mu\text{M}$			
(b)			
[G4G4G3G-MU] (mM)	k_{obs} (s ⁻¹)	$\Delta P/E_0$	v_{ss}/E_0 (s ⁻¹)
1.97	53 ± 4	0.048	0.20
1.29	50 ± 2	0.046	0.20
0.985	44 ± 3	0.043	0.21
0.493	37 ± 3	0.031	0.18
0.246	40 ± 3	0.026	0.12
0.123	33 ± 3	0.021	0.078
0.062	30 ± 3	0.016	0.040
0.031	17 ± 6	0.006	0.024
$E_0 = 8.9 \mu\text{M}$			
(c)			
[G4G4G4G3G-MU] (mM)	k_{obs} (s ⁻¹)	$\Delta P/E_0$	v_{ss}/E_0 (s ⁻¹)
0.835	41 ± 2	0.044	0.22
0.556	45 ± 2	0.034	0.20
0.418	48 ± 3	0.026	0.18
0.209	36 ± 3	0.022	0.12
0.104	39 ± 4	0.013	0.074
0.052	45 ± 7	0.009	0.044
0.026	43 ± 13	0.004	0.02
$E_0 = 8.9 \mu\text{M}$			

Table 3 Steady-state kinetic parameters (30 °C, pH 7.2, / 0.15) calculated from the v_{ss}/E_0 values of Table 1

Compound	k_{cat}/K_m (mM ⁻¹ · s ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	K_i (mM)
G4G3G-MU	0.46 ± 0.05	0.5 ± 0.2	1.0 ± 0.2	6 ± 1.5
G4G3G-3,4-DNP	11 ± 0.5	7.8 ± 1.4	0.7 ± 0.2	0.9 ± 0.7
G4G3G-2,4-DNP	500 ± 80	400 ± 100	0.7 ± 0.2	3.5 ± 2
G4G4G3G-MU	0.8 ± 0.05	0.4 ± 0.1	0.5 ± 0.1	2.5 ± 0.6
G4G4G4G3G-MU	0.8 ± 0.07	0.6 ± 0.1	0.6 ± 0.2	1.4 ± 0.5

species, so the aglycone, as well as the length of the oligomer, is of little importance ($n = 2-4$).

The amount of aglycone released in the pre-steady-state phase is determined by the integral of dP/dt in the time range where the productive enzyme–substrate complex changes from the initial amount after the lag phase, ES_0 , to the final steady-state amount, ES_{ss} . That is:

$$ES(t) = ES_{\text{ss}} + (ES_0 - ES_{\text{ss}}) \cdot \exp[-k_{\text{obs}}(t)] \quad (3)$$

The integration leads to the amount of product, ΔP , released in the pre-steady-state phase:

$$\Delta P = (k_{\text{cat}})_0 \cdot (ES_0 - ES_{\text{ss}}) / k_{\text{obs}} \quad (4)$$

Figure 3 Analysis of steady-state rate data

In this example, the solid line represents the curve with fitted parameter values: $K_m = 0.7$ mM, $K_i = 0.9$ mM and $k_{\text{cat}} = 7.8$ s⁻¹ (Table 3) and the broken line represents the curve with fitted parameter values: $K_m = 1.6 \pm 0.2$ mM, $K_i = 0.31 \pm 0.05$ mM and $k_{\text{cat}} = 16 \pm 2$ s⁻¹.

Table 4 Kinetic parameters (30 °C, pH 7.2, / 0.15) calculated from the v_0/E_0 values [eqn (5)*]

Compound	$(k_{\text{cat}}/K_m)_0$ (mM ⁻¹ · s ⁻¹)	$(k_{\text{cat}})_0$ (s ⁻¹)	$(K_m)_0$ (mM)	$k_{\text{cat}}/K_m^\dagger$ (mM ⁻¹ · s ⁻¹)
G4G3G-MU	6.5 ± 0.4	7 ± 0.9	1.1 ± 0.3	0.46 ± 0.05
G4G3G-3,4-DNP	100 ± 6	43 ± 3.4	0.4 ± 0.07	11 ± 0.5
G4G3G-2,4-DNP	1120 ± 100	> 2000	> 2	500 ± 80
G4G4G3G-MU	5.8 ± 0.3	4 ± 0.5	0.7 ± 0.2	0.8 ± 0.05
G4G4G4G3G-MU	6.1 ± 0.3	3.3 ± 0.15	0.54 ± 0.05	0.8 ± 0.07

*, using v_{ss} data calculated for absent substrate inhibition; †, steady-state values.

where $(k_{\text{cat}})_0$ is k_{cat} before the change occurs. The series of substrates with different leaving groups represents steady-state k_{cat} values differing roughly 100 times, and ΔP is clearly proportional to these (Tables 1 and 2). Also as expected, ΔP is found to be proportional to the total enzyme concentration (results not shown). In agreement with previous results [3], we find that ΔP is not a measure of a burst of product concomitant with the formation of a slowly converting enzyme intermediate, as seen clearly from the results on the G4G3G-2,4-DNP-substrate (6), where ΔP corresponds to a concentration of hydrolysed substrate up to approx. 30 times greater than the total concentration of enzyme, E_0 (Table 1). If we look at the initial rate of fast production of product, $v_0 = (k_{\text{cat}})_0 \cdot (ES_0)$. From eqn (4):

$$v_0 = (k_{\text{cat}})_0 \cdot (ES_0) = k_{\text{obs}} \cdot \Delta P + v_{\text{ss}} \quad (5)$$

The substrate concentration dependencies of v_0 values calculated according to eqn (5) [neglecting the substrate inhibition in the steady state, i.e. calculating the rates, v_{ss} , from $k_{\text{cat}} \cdot E_0 / (1 + K_m/S)$, using the parameter values of Table 3] showed good agreement with the Michaelis–Menten equation and the values of the kinetic parameters are given in Table 4. It is seen that $(k_{\text{cat}}/K_m)_0$ is always bigger than (k_{cat}/K_m) . This means, however, that the proposed scheme (Scheme 3 in [3]) cannot explain the results. According to this scheme, the change from fast to slow

Scheme 1 Suggested reaction scheme of 1,3-1,4- β -glucanase catalysis

product formation is due to a change of the active ES-complex into inactive ES^* and ES_2^* complexes, the values of k_{cat} and K_m are affected exactly the same when the inactive species are formed, so the original scheme predicts that $(k_{cat}/K_m)_0 = (k_{cat}/K_m)$. An observed effect on (k_{cat}/K_m) usually means that enzyme species do not have a substrate bound productively, e.g. an E^* with the same conformation of the enzyme as ES^* should be taken into consideration. However, this is not the solution here. E and E^* would be equilibrated before mixing and equilibration of ES^*/ES would happen concomitantly with their formation, so that no pre-steady-state phase would be observed. We therefore suggest a new scheme (Scheme 1) to explain the results.

Scheme 1 includes a rather tight ($K_3 \ll K_4$) non-productive, non-inhibitory enzyme–substrate interaction, SE , that saturates a regulatory site at low substrate concentration. SE is formed and reacts productively with S in the lag phase. The relatively slow conversion of E' into E governs the rate of this part of the reaction. The SE and the productive SES complex undergo slow conformational changes corresponding to k_{obs} and form the SE^* (non-productive) and the less active (or inactive) SE^*S . Since substrate inhibition is observed in the steady state, a third inhibitory substrate binding is also included.

According to Scheme 1, the fast production of the aglycone, corresponding to v_0 after the lag phase and before any SE^* or SE^*S is formed is:

$$v_0 = k_{+2} \cdot E_0 / \{1 + K_4/S \cdot [1 + K_3/S \cdot (1 + K_0)]\} \quad (6)$$

where $k_{+2} = (k_{cat})_0$ and $E'/E = K_0$.

When $S \gg K_3$ and $K_4 \gg K_3$, as we assume, eqn (6) becomes:

$$v_0 \cong k_{+2} \cdot E_0 / (1 + K_4/S) \quad (7)$$

The corresponding steady state rate, v_{ss} , (not taking the substrate inhibition into consideration) is:

$$v_{ss} = k_{cat} \cdot E_0 / (1 + K_m/S) \quad (8)$$

where $k_{cat} = k_{+2}/(1 + K_7)$ and $K_m = K_4(1 + K_5)/(1 + K_7)$,

Table 5 Kinetic parameters (30 °C, pH 7.2, I 0.15) calculated from eqns (8) and (9)

Compound	K_5	K_7
G4G3G-MU	14 ± 2	14 ± 6
G4G3G-3,4-DNP	9 ± 1	6 ± 1
G4G3G-2,4-DNP	2 ± 0.5	> 5
G4G4G3G-MU	7 ± 0.5	10 ± 3
G4G4G4G3G-MU	8 ± 0.5	6 ± 2

so that:

$$(k_{cat}/K_m)_0 = (k_{cat}/K_m)_{ss} \cdot (1 + K_5) \quad (9)$$

and

$$(k_{cat})_0 = (k_{cat})_{ss} \cdot (1 + K_7) \quad (10)$$

Table 5 shows the K_5 and K_7 values calculated according to eqns (9) and (10). Because of the corrections for substrate inhibition, the values of K_7 are less reliable, than those of K_5 , but apparently the K_5 and K_7 values are not greatly different.

According to Scheme 1, the pre-steady-state change from fast to slow product formation is a result of the accumulation of the inactive enzyme species SE^* and SE^*S and also, when substrate inhibition occurs, SE^*S_2 . The observed rate constant, k_{obs} , then corresponds to the relaxation time of steps 5 and 7, and may be deduced as follows. The change in SE^* and SE^*S is:

$$d(SE^* + SE^*S)/dt = k_{+5} \cdot SE + k_{+7} \cdot SES - k_{-5} \cdot SE^* - k_{-7} \cdot SE^*S \quad (11)$$

if we add and subtract $k_{-7} \cdot SES$ and $k_{-5} \cdot SE$, we get:

$$d(SE^* + SE^*S)/dt = (k_{+5} + k_{-5})SE + (k_{+7} + k_{-7})SES - [k_{-5}(SE^* + SE) + k_{-7}(SE^*S + SES)] \quad (12)$$

We have assumed that $S \gg K_3$, so that $(E' + E) \ll SE$, so the total concentration of enzyme, E_{Total} , is given by:

$$E_{Total} = SE^* + SE + SE^*S + SES \quad (13)$$

then:

$$d(SE^* + SE^*S)/dt = -d(SE + SES)/dt \quad (14)$$

If it is further assumed that step 4 is fast compared with steps 5 and 7, we have in the time range after the lag phase:

$$SE/SES = K_4/S \quad (15)$$

and:

$$(SE + SES) = SES(1 + K_4/S) = SE(1 + S/K_4) \quad (16)$$

Combining eqns (12) and (16) leads to:

$$-d(SE + SES)/dt = \{[(k_{+5} + k_{-5})/(1 + S/K_4)] + [(k_{+7} + k_{-7})/(1 + K_4/S)]\}(SE + SES) - [k_{-5}(SE^* + SE) + k_{-7}(SE^*S + SES)] \quad (17)$$

Table 6 pH-dependence of kinetic parameters of the G4G3G-3,4-DNP reaction at 30 °CUsing v_{ss} data calculated for absent substrate inhibition.

pH	$(k_{cat}/K_m)_0$ (mM ⁻¹ · s ⁻¹)	$(k_{cat})_0$ (s ⁻¹)	$(K_m)_0$ (mM)	k_{cat}/K_m (mM ⁻¹ · s ⁻¹)
6.23	135	41	0.3	15
6.76	150	45 ± 3.5	0.3 ± 0.1	17 ± 2
7.20	110 ± 6	42 ± 3.4(35)	0.4 ± 0.07(0.35)	11.6
7.80	45	18	0.4	4
8.25	17	—	—	2.08

Figure 4 Hydrogen-ion saturation curve of the $(k_{cat}/K_m)_0$ parameter values of G3G4G-3,4-DNP-catalysed hydrolysis in the pH range 6.76–8.25

If the term $[k_{-5}(SE^* + SE) + k_{-7}(SE^*S + SES)]$ is reasonably constant, that is if $k_{-5} \cong k_{-7}$, as seen from eqn (13), eqn (17) is a simple first-order differential equation and we obtain:

$$k_{obs} = [(k_{+5} + k_{-5})/(1 + S/K_4) + (k_{+7} + k_{-7})/(1 + K_4/S)] \quad (18)$$

It may be seen rather easily that, with $K_5 \cong K_7$ (Table 5), the observed relatively constant value of k_{obs} can be explained. At low substrate concentration, $S < K_4$, the major term of k_{obs} is $(k_{+5} + k_{-5})/(1 + S/K_4)$ due to the conversion of SE, leading to $k_{obs} \cong (k_{+5} + k_{-5})$, and at high substrate concentration $S > K_4$, the major term of k_{obs} is $(k_{+7} + k_{-7})/(1 + K_4/S)$ due to the conversion of SES, leading to $k_{obs} \cong (k_{+7} + k_{-7})$. Thus Scheme 1 explains the stopped-flow kinetic results.

The pH-dependence of the β -glucanase catalysed hydrolysis of G4G3G-3,4-DNP was also investigated using the stopped-flow technique. This substrate was chosen because it showed relatively weak substrate inhibition and rates in the most suitable range for analysis. Table 6 shows the results obtained according to Scheme 1 and Figure 4 shows the proton-saturation curve corresponding to the $(k_{cat}/K_m)_0$ pH dependence. The resulting pK value of the SE-enzyme species (Scheme 1) is 7.2 ($K = 60 \pm 6.7$ nM), in good agreement with the value that may be expected for a glutamic acid residue working as an acid catalyst (Glu¹³⁸ [27]). $(K_m)_0$ is only slightly affected up to pH 7.8, but becomes too large to be determined at pH 8.25 (Table 6). The pH dependence of $(k_{cat}/K_m)_0$, the kinetic parameter determined at low substrate concentrations corresponding to free enzyme species, thus differs from that of $(K_m)_0$. A shift of the pK value of the

glutamic acid residue to a value greater than 8 seems unlikely, so apparently another ionizing group is also affecting the kinetics. The balance between the two conformations, SE and SE*, is rather independent of pH in this range, K_5 does not change significantly, the same seems to be true for SES and SE*S, but, as discussed above (Figure 3), the estimates of the k_{cat} values are not sufficiently reliable.

We have obtained an important insight into the regulation of β -glucanase action. The enzyme cleaves polysaccharide components of the cell walls of the higher plant family Poaceae. Structurally, they are linear glucans of up to 1200 β -D-glucosyl residues linked through β -1,3 and β -1,4 bonds and the products of the enzymic reaction are mainly G4G3G and G4G4G3G. Scheme 1 suggests the possible switch between several conformations of the enzyme. Activation coupled with substrate binding at a regulatory site (the E', E, SE pathway) leading to the most active species, SES, a mechanism that corresponds to an allosteric activator mechanism of substrate binding, can be an effective regulatory mechanism, particularly of enzymes cleaving polymeric substrates, since the enzyme is kept attached to the substrate after cleavage and further interactions are facilitated. It is more difficult to understand the strong tendency to relaxation into the inactive or less active species, SE* and SE*S. One possibility is that the small oligosaccharide substrates that we have investigated in the present study, which show this negative regulatory effect on the enzyme after the initiation of the reaction, serve as feedback regulatory inhibitors of the hydrolysis and that a polymeric substrate would not immediately show this behaviour. The link between the regulatory and the reactive site of polymeric substrates may help to keep the enzyme in its most active conformation.

CONCLUSION

To explain the pre-steady-state kinetic results on β -glucanase reactions obtained in the present study, we have had to suggest a rather complicated mechanism of substrate binding (Scheme 1). Apparently, the free enzyme exists in two conformations, one of which binds oligomeric substrates, much resembling the natural products of hydrolysis of β -glucans, rather strongly in a regulatory site, before any productive interactions can take place. This corresponds to an allosteric activation mechanism. With these substrates, however, the productive enzyme species are also able to change into less active or inactive forms, which may be seen as a feedback inhibitory mechanism. How the polymeric substrate is using the regulatory possibilities, and if, perhaps, it is able to keep the enzyme in its active form due to the link between the two interacting parts of the substrate, has yet to be investigated.

REFERENCES

- Henrissat, B. (1991) A classification of glycosyl hydrolases based on amino acid sequence similarity. *Biochem. J.* **280**, 309–316.
- Coutinho, P. M. and Henrissat, B. (1999) Carbohydrate-active enzymes: an integrated database approach. In *Recent Advances in Carbohydrate Bioengineering* (Gilbert, H. J., Davies, G. J., Henrissat, B. and Svensson, B., eds.), pp. 3–12. The Royal Society of Chemistry, Cambridge.
- Abel, M., Planas, A. and Christensen, U. (2001) Presteady-state kinetics of *Bacillus* 1,3-1,4- β -glucanase: binding and hydrolysis of a 4-methylumbelliferyl trisaccharide substrate. *Biochem. J.* **357**, 195–202.
- Sinnott, M. L. (1990) Catalytic mechanisms of enzymic glycosyl transfer. *Chem. Rev.* **90**, 1171–1202.
- Davies, G., Sinnott, M. L. and Withers, S. G. (1998) Glycosyl transfer. In *Comprehensive Biological Catalysis* (Sinnott, M. L., ed.), pp. 119–209. Academic Press Ltd, San Diego.
- Withers, S. G. (2001) Mechanism of glycosyl transferases and hydrolases. *Carbohydr. Polym.* **44**, 325–337.

- 6 Heightman, T. D. and Vasella, A. (1999) Recent insights into inhibition, structure, and mechanism of configuration-retaining glycosidases. *Angew. Chem. Int. Ed. Engl.* **38**, 750–770
- 7 Tanaka, A., Ito, M. and Hiromi, K. (1986) Equilibrium and kinetic studies on the binding of gluconolactone to almond β -glucosidase in the absence and presence of glucose. *J. Biochem.* **100**, 1379–1385
- 8 Maguire, R. J. (1977) Kinetics of the hydrolysis of cellobiose and *p*-nitrophenyl- β -D-glucoside by cellobiase of *Trichoderma viride*. *Can. J. Biochem.* **55**, 19–26
- 9 Tanaka, A., Ohnishi, M. and Hiromi, K. (1982) Stopped-flow kinetic studies on the binding of gluconolactone and maltose to glucoamylase. *Biochemistry* **21**, 107–113
- 10 Olsen, K., Svensson, B. and Christensen, U. (1992) Stopped-flow fluorescence and steady-state kinetic studies of ligand-binding reactions of glucoamylase from *Aspergillus niger*. *Eur. J. Biochem.* **209**, 777–784
- 11 Olsen, K., Christensen, U., Sierks, M. R. and Svensson, B. (1993) Reaction mechanisms of Trp¹²⁰→Phe and wild-type glucoamylases from *Aspergillus niger*. Interactions with maltooligodextrins and acarbose. *Biochemistry* **32**, 9686–9693
- 12 Christensen, U., Olsen, K., Stoffer, B. B. and Svensson, B. (1996) Substrate binding mechanism of Glu¹⁸⁰→Gln, Asp¹⁷⁶→Asn, and wild-type glucoamylases from *Aspergillus niger*. *Biochemistry* **35**, 15009–15018
- 13 Christensen, U. (2000) pH-dependence of the fast step of maltose hydrolysis catalyzed by glucoamylase G1 from *Aspergillus niger*. *Biochem. J.* **349**, 623–628
- 14 Tull, D. and Withers, S. G. (1994) Mechanisms of cellulases and xylanases: a detailed kinetic study of the exo- β -1,4-glycanase from *Cellulomonas fimi*. *Biochemistry* **33**, 6363–6370
- 15 Zechel, D. L., Konermann, L., Withers, S. G. and Douglas, D. J. (1998) Presteady-state kinetic analysis of an enzymatic reaction monitored by time-resolved electrospray mass spectrometry. *Biochemistry* **37**, 7664–7669
- 16 van Tilbeurgh, H., Loontjens, F. G., Engelborgs, Y. and Claeysens, M. (1989) Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Binding of small ligands to the 1,4- β -glucan cellobiohydrolase II and influence of glucose on their affinity. *Eur. J. Biochem.* **184**, 553–559
- 17 Johnson, K. A. (1992) Transient-state kinetic analysis of enzyme reaction pathways. In *The Enzymes* (Sigman, D. S., ed.), 3rd edn, vol. **22**, pp. 1–61, Academic Press, San Diego
- 18 Planas, A. (2000) Bacterial 1,3-1,4- β -glucanases: structure, function and protein engineering. *Biochim. Biophys. Acta* **1543**, 361–382
- 19 Hrmova, M. and Fincher, B. (2001) Structure–function relationships of β -D-glucan endo- and exohydrolases from higher plants. *Plant Mol. Biol.* **47**, 73–91
- 20 Anderson, M. A. and Stone, B. A. (1975) A new substrate for investigating the specificity of β -glucan hydrolases. *FEBS Lett.* **52**, 202–207
- 21 Malet, C., Jiménez-Barbero, J., Bernabé, M., Brosa, C. and Planas, A. (1993) Stereochemical course and structure of the products of the enzymic action of endo-1,3-1,4- β -D-glucan 4-glucanohydrolase from *Bacillus licheniformis*. *Biochem. J.* **296**, 753–758
- 22 Keitel, T., Simon, O., Borriess, R. and Heinemann, U. (1993) Molecular and active site structure of a *Bacillus* 1,3-1,4- β -glucanase. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5287–5291
- 23 Hahn, M., Pons, J., Planas, A., Querol, E. and Heinemann, U. (1995) Crystal structure of *Bacillus licheniformis* 1,3-1,4- β -D-glucan 4-glucanohydrolase at 1.8 Å resolution. *FEBS Lett.* **374**, 221–224
- 24 Malet, C. and Planas, A. (1997) Mechanism of *Bacillus* 1,3-1,4- β -D-glucan 4-glucanohydrolases: kinetics and pH studies with 4-methylumbelliferyl β -glucan oligosaccharides. *Biochemistry* **36**, 13838–13848
- 25 Planas, A., Millet, O., Palasí, J., Pallarés, C., Abel, M. and Viladot, J. L. (1998) Synthesis of aryl 3-O- β -cellobiosyl- β -D-glucopyranosides for reactivity studies of 1,3-1,4- β -glucanases. *Carbohydr. Res.* **310**, 53–64
- 26 Reference deleted
- 27 Viladot, J. L., de Ramon, E., Durany, O. and Planas, A. (1998) Probing the mechanism of *Bacillus* 1,3-1,4- β -D-glucan 4-glucanohydrolases by chemical rescue of inactive mutants at catalytically essential residues. *Biochemistry* **37**, 11332–11342
- 28 Pons, J., Querol, E. and Planas, A. (1997) Mutational analysis of the major loop of *Bacillus* 1,3-1,4- β -D-glucan 4-glucanohydrolases. Effects on protein stability and substrate binding. *J. Biol. Chem.* **272**, 13006–13012
- 29 Planas, A. (1998) Mutational analysis of specificity and catalysis in *Bacillus* 1,3-1,4- β -glucanases. In *Carbohydrases from Trichoderma reesei and other microorganisms* (Claeysens, M., Nerinckx, W. and Piens, K., eds.), pp. 21–38, The Royal Society of Chemistry, Cambridge
- 30 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 31 Gill, S. C. and van Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319–326
- 32 Malet, C., Viladot, J. L., Ochoa, A., Gállego, B., Brosa, C. and Planas, A. (1995) Synthesis of 4-methylumbelliferyl- β -D-glucan oligosaccharides as specific chromophoric substrates of 1,3-1,4- β -D-glucan 4-glucanohydrolases. *Carbohydr. Res.* **274**, 285–301
- 33 Piotukh, K., Serra, V., Borriess, R. and Planas, A. (1999) Protein–carbohydrate interactions defining substrate specificity in *Bacillus* 1,3-1,4- β -D-glucan 4-glucanohydrolases as dissected by mutational analysis. *Biochemistry* **38**, 16092–16104