Hydrolyses of α- and β-cellobiosyl fluorides by Cel6A (cellobiohydrolase II) of *Trichoderma reesei* and *Humicola insolens*

Dieter BECKER*, Karin S. H. JOHNSON*, Anu KOIVULA†, Martin SCHÜLEIN‡ and Michael L. SINNOTT*[1]

*Department of Paper Science, UMIST, P. O. Box 88, Sackville Street, Manchester M60 1QD, U.K., †VTT Biotechnology and Food Research, P.O. Box 1500, FIN-02044 VTT, Espoo, Finland, and ‡Novo-Nordisk A/S, Novo Alle, DK-2880, Bagsværd, Denmark

We have measured the hydrolyses of α- and β-cellobiosyl fluorides by the Cel6A [cellobiohydrolase II (CBHII)] enzymes of *Humicola insolens* and *Trichoderma reesei*, which have essentially identical crystal structures [Varrot, Hastrup, Schülein and Davies (1999) Biochem. J. **337**, 297–304]. The β-fluoride is hydrolysed according to Michaelis–Menten kinetics by both enzymes. When the ~ 2.0 % of β-fluoride which is an inevitable contaminant in all preparations of the α-fluoride is hydrolysed by Cel7A (CBHI) of *T. reesei* before initial-rate measurements are made, both Cel6A enzymes show a sigmoidal dependence of rate on substrate concentration, as well as activation by cellobiose. These kinetics are consistent with the classic Hehre resynthesis–hydrolysis mechanism for glycosidase-catalysed hydrolysis of the 'wrong' glycosyl fluoride for both enzymes. The Michaelis–Menten kinetics of α-cellobiosyl fluoride hydrolysis by the *T. reesei* enzyme, and its inhibition by cellobiose, previously reported [Konstantinidis, Marsden and Sinnott (1993) Biochem. J. **291**, 883–888] are withdrawn. ^{1}H NMR monitoring of the hydrolysis of α-cellobiosyl fluoride by both enzymes reveals that in neither case is α-cellobiosyl fluoride released into solution in detectable quantities, but instead it appears to be hydrolysed in the enzyme active site as soon as it is formed.

Key words: co-operativity, Hehre mechanism, transglycosylation.

INTRODUCTION

Glycosidases catalyse the hydrolysis of glycosidic bonds with either retention or inversion of the anomeric configuration. All inverting glycosidases so far discovered appear to operate by the single-displacement mechanism first suggested in outline by Koshland [1]: nucleophilic attack by water at the anomeric centre, assisted by partial proton removal from the water (general base catalysis) and partial proton donation to the aglycone leaving group (general acid catalysis) by aspartate or glutamate residues on the protein [2,3] (cf. Scheme 1, left panel). The best evidence for the details of this mechanism lies in a series of elegant experiments by E. J. Hehre and collaborators. Starting about 20 years ago [4], they showed that inverting glycosidases could hydrolyse the glycosyl fluorides of opposite configuration to the substrate by a resynthesis–hydrolysis mechanism involving two molecules of the 'wrong' fluoride in the first step, in which the departing fluorine of the 'wrong' fluoride occupies the position of the nucleophilic water, and a reverse protonated form of the enzyme is the active species (cf. Scheme 1, right panel). The kinetic features of this mechanism included sequential two-substrate kinetics (with both substrates the same), rather than simple one-substrate Michaelis–Menten kinetics, and the ability of another saccharide to replace the second fluoride molecule. In favourable cases, the initial transfer product would be released from the enzyme, and its build-up in free solution could be observed [5].

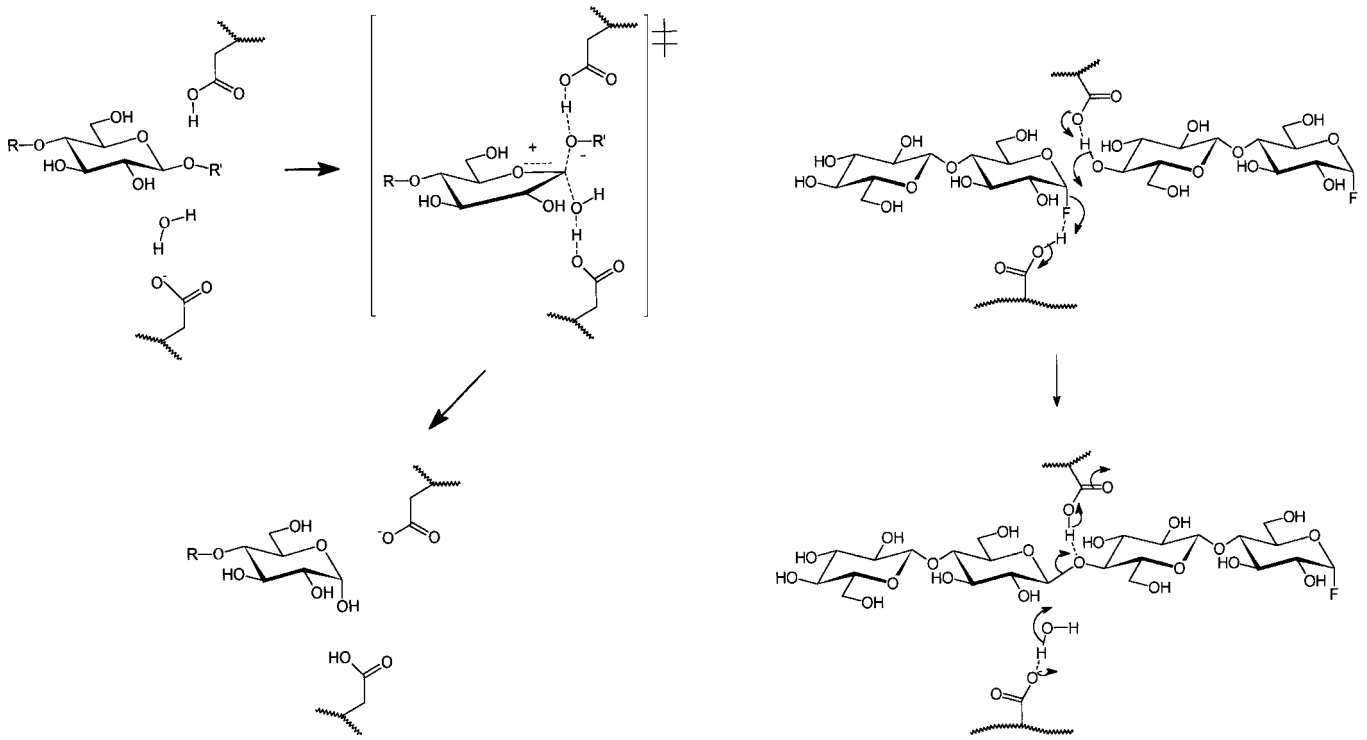
Over the last decade, the classification of glycosidase sequences into families has greatly simplified consideration of glycosidase mechanism (latest hard-copy update, [6]; continuously updated website, http://afmb.cnrs-mrs.fr/~pedro/CAZY/). The classification among families has a perfect correlation with enzyme stereochemistry, although details of the acid–base catalytic machinery can differ, at least within families in clan GH-A. The usual glutamate acid/base in families 1 and 2 is replaced in myrosinase (family 1) by a glutamine [7], and by a Mg^{2+} co-ordinated to the glutamate in the *Escherichia coli* β-galactosidases (family 2) [2]. Family 6 comprises enzymes that hydrolyse β(1 → 4) glucan linkages with inversion of the configuration, and includes both cellobiohydrolases [cellobiohydrolase II (CBHII)] and inverting endoglucanases. The crystal structures of endoglucanase E2 from *Thermomonospora fusca* [8] and of two cellobiohydrolases {Cel6A (CBHII) from *Trichoderma reesei* [9] and *Humicola insolens* [10]} have been solved, with the protein fold as expected [11] in all four enzymes being very similar, and with the cellobiohydrolases differing from the endoglucanases in the presence of loop of protein forming a lid over the active-site cleft, converting it into a tunnel.

The catalytic mechanism of all four enzymes acting on a β-cellobioside is likely to be that in shown in Scheme 1 (left panel), and the action on α-cellobiosyl fluoride is likely to be that in Scheme 1 (right panel), although there is evidence that the reactive conformation of the ring in the −1 site may not be the $^4C_1$ conformation shown for simplicity in these schemes [12]. The Hehre mechanism for the hydrolysis of α-cellobiosyl fluoride by the endoglucanase CenA of *Cellulomonas fimi*, another enzyme of family 6, has been established previously [13]. However, the X-ray crystal structure of Cel6A (CBHII) of *Trichoderma reesei* [9] appeared to reveal no likely candidate for the catalytic base: Glu-401, which is conserved in all family 6 enzymes, is involved in salt-bridge interactions which were interpreted as preventing it acting catalytically. Nonetheless, the homologue of Glu-401 in *C. fimi* CenA was shown clearly to be the active-site base by site-directed mutagenesis experiments [14].

The hydrolysis of α-cellobiosyl fluoride by Cel6A of *T. reesei* was investigated initially in the lab of one of the authors (M. L. S.) in the expectation merely that another example of the Hehre mechanism would be uncovered. We could, though, detect no

Abbreviation used: CBHII, cellobiohydrolase II.

[1] To whom correspondence should be addressed (e-mail Michael.Sinnott@umist.ac.uk).



Scheme 1 Mechanism of hydrolysis of a β(1 → 4) glucan link by an inverting glucanase (left panel), and Hehre mechanism for the transformation of α-cellobiosyl fluoride by Cel6 enzymes (right panel)

build-up of transfer products, and the hydrolysis appeared to conform to Michaelis–Menten kinetics and be inhibited by cellobiose; we reported this and proposed a novel mechanism [15].

We now report that when care is taken to selectively hydrolyse the ~ 2% contaminant of β-anomer (which is an inescapable contaminant in all freshly prepared samples of α-cellobiosyl fluoride) before any measurements of its hydrolysis by Cel 6A are made, the hydrolyses by the Cel6A enzymes of *T. reesei* and of *H. insolens* exhibit kinetics entirely in accord with the Hehre mechanism. However, when the hydrolysis of α-cellobiosyl fluoride by both enzymes is monitored to completion by ^{1}H NMR, with neither enzyme is a build-up of cellotetraosyl fluoride detectable, as reported previously for the *T. reesei* enzyme.

EXPERIMENTAL

α- and β-Cellobiosyl fluorides were synthesized essentially as described previously [15], although HF/pyridine (7:3, v/v) [16,17] rather than liquid HF was used to convert octa-acetyl-cellobiose into hepta-acetyl-α-cellobiosyl fluoride. Fluoride ion liberation was followed essentially as described previously [15]. The reaction was carried out in a stirred container with an integral jacket through which water maintained at 25.0 °C was circlated from a Haake temperature bath. Data from a combination fluoride ion electrode (Corning or Orion) were collected via a Mettler Delta 350 pH-ion meter and a serial interface by an IBM-compatible PC, using the software Hyperterminal for recording. Data were analysed using the PC software FigP. Prior to the determination of initial rates, the β-cellobiosyl fluoride contaminant present in all preparations of its anomer was hydrolysed by *T. reesei* CBHI; this contamination was 1.8% in the preparation used for the *T. reesei* experiments and 2.4% in that used in the *H. insolens* experiments. In the case of β-cellobiosyl fluoride, the level of substrate degradation after deacetylation was tested with the fluoride ion probe by comparing fully hydrolysed substrate (using *T. reesei* Cel6A for degradation) with the starting material. Batches showing over 6% degradation were not used for kinetic experiments. Levels of free fluoride present in the final deacetylated product were less than 1% for the α-fluoride and 3.6% for the β-fluoride.

Hydroxide ion interferes with the response of the fluoride ion electrode, and therefore kinetic studies for both enzymes were performed in 0.1 M sodium acetate buffer, pH 5.0. This is some distance away from the pH optima of the *Humicola* enzymes [18].

The hydrolysis of α-cellobiosyl fluoride was measured with 5.4 μM CBHII from *H. insolens* and 0.67 μM CBHII from *T. reesei*, and that of β-cellobiosyl fluoride with 1.2 μM CBHII from *H. insolens*.

The complete hydrolysis of α-cellobiosyl fluoride (8.3 mM) in ^2H_2O (Aldrich) was monitored by ^{1}H NMR, using a Bruker Avance/DPX 400 instrument to record 16 scans per spectrum; the probe temperature was 20 °C. The *H. insolens* enzyme was obtained as a lyophilized solid and used directly at a concentration of 12 mg/ml in ^2H_2O buffered with 0.1 M Mes (pH meter reading 6.0). The *T. reesei* enzyme was obtained as a 90 μM solution and was exchanged five times with 0.1 M sodium acetate buffer in ^2H_2O (pH-meter reading 5.01), using Ultrafree centrifugal protein concentrators with polyether sulphone membrane and a 30 kDa cut-off, to give a final concentration of 0.48 mg/ml.

RESULTS AND DISCUSSION

Figure 1 shows the initial rate of fluoride ion release from α-cellobiosyl fluoride by the Cel6A enzymes of *T. reesei* and of *H.*

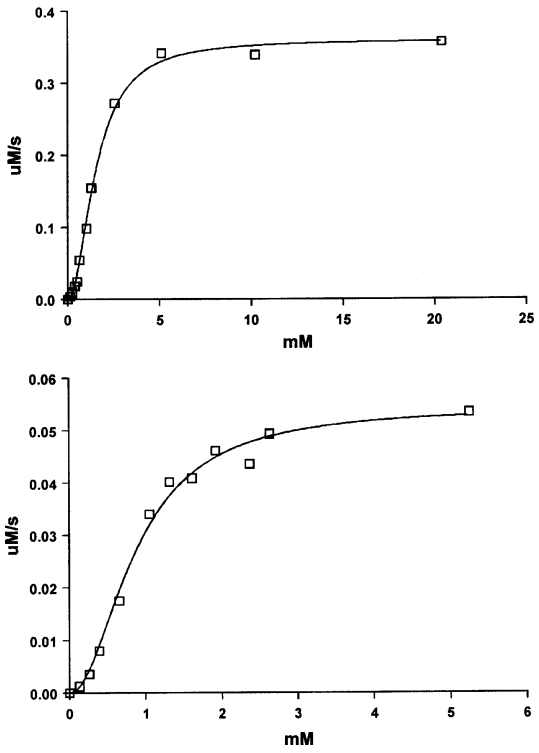
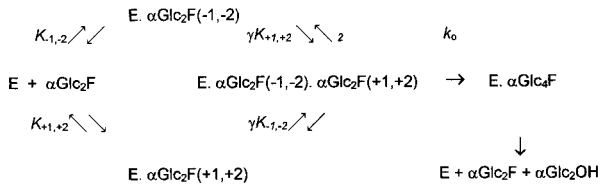


Figure 1 Variation with substrate concentration of the rate of liberation of fluoride ion from α-cellobiosyl fluoride by *T. reesei* Cel6A (0.67 μM) (upper panel) and *H. insolens* Cel6A (5.4 μM) (lower panel)

Reactions were carried out in 0.1 M sodium acetate buffer, pH 5.0, at 25 °C. Fluoride ion liberation is given in units of μM/s.



Scheme 2 Suggested kinetic pathway for the transformation of α-cellobiosyl fluoride by the CBHII enzymes of *T. reesei* and *H. insolens*

E denotes enzyme; numbers in parentheses refer to the binding sites of the sugar residues; and γ is the proportionality constant governing the ratio of binding constants.

insolens as a function of substrate concentration, when care is taken to hydrolyse contaminating β-fluoride using Cel7A (CBHI) of *T. reesei* before initial rates are measured. It is clear that the kinetics are not hyperbolic, but sigmoidal. If the Hehre molecular mechanism applies, one expects the random sequential kinetic mechanism of Scheme 2 to apply. The numbers in parentheses refer to the binding sites of the sugar residues: since cellobiose is not a substrate, we neglect binding across the cleavage site (i.e. $-1,+1$).

Because of the unnatural nature of the transglycosylation step, and the reverse-protonated form of the enzyme needed to bring it about, in the first instance we assume that all steps subsequent to the transglycosylation are fast, as are the binding steps and their reversal preceding it. The rate law for the resulting rapid-equilibrium random mechanism then becomes that of eqn. (1) (where E denotes enzyme):

$$V = \frac{k_o[E]_o[\alpha Glc_2F]^2}{\gamma K_{-1,-2}K_{+1,+2} + \gamma(K_{-1,-2} + K_{+1,+2})[\alpha Glc_2F] + [\alpha Glc_2F]^2} \quad (1)$$

Note that, in order to avoid confusion with α as a designator of anomeric stereochemistry, the proportionality constant governing the ratio of binding constants in the thermodynamic box of Scheme 2 is designated γ, rather than the conventional α. Eqn. (1) permits the determination of only three independent parameters rather than four, and can be rewritten as eqn. (2), where S is αGlc_2F:

$$v = a[S]^2/(b + c[S] + [S]^2) \quad (2)$$

Attempts to fit the data for both enzymes to eqn. (2) gave values for c of 0, within experimental error. This means that complexes of enzyme and a single cellobiosyl fluoride molecule cannot be detected. Within the resolution of our experiments, the system is infinitely co-operative: the affinity of the complex of the enzyme with one cellobiosyl fluoride for the second cellobiosyl fluoride molecule is very much greater than the affinity of the unliganded enzyme for the first cellobiosyl fluoride molecule. We accordingly fitted the data to the two-parameter model of eqn. (3), corresponding physically to a situation where only complexes of enzyme and two cellobiosyl fluoride molecules are important:

$$v = k_{cat}[E]_o[S]^2/(K_{m2} + [S]^2) \quad (3)$$

These are the fits shown in Figure 1. k_{cat} has its normal meaning, and in the case of a rapid-equilibrium mechanism K_{m2} is the dissociation constant of the complex of enzyme and two cellobiosyl fluoride molecules into free enzyme and cellobiosyl fluoride. Derived data for the hydrolysis of both fluorides by both enzymes are given in Table 1; the original data of Konstantinidis et al. [15] on the β-fluoride hydrolysed by the *T. reesei* enzyme were confirmed. It is noteworthy that k_{cat} for the incorrect α-fluoride is a mere 7–8-fold lower than that for the β-fluoride; given the 40-fold higher spontaneous rate of hydrolysis of β-glucosyl fluoride than of α-glucosyl fluoride [19], this implies that transition-state interactions in the $+1$ and $+2$ sites more than compensate for the change of anomeric configuration and the use of the reverse-protonated form of the enzyme in catalysis.

The action of cellobiose was also investigated. At low substrate concentrations cellobiose should always activate, since there is no competition between α-cellobiosyl fluoride and cellobiose for the $-2,-1$ sites, which must be occupied by α-cellobiosyl fluoride rather than cellobiose if a reaction is to occur. At concentrations close to saturation, however, if cellobiose has a higher affinity than α-cellobiosyl fluoride for the $-2,-1$ sites,

Table 1 Hydrolysis of cellobiosyl fluorides in 0.1 M sodium acetate buffer, pH 5.0, at 25.0 °C

	β-Fluoride		α-Fluoride	
Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_{m2} (mM^2)
H. insolens	0.071	0.022	0.010	0.77 ± 0.08
T. reesei	4.0	0.15	0.54	2.35 ± 0.15

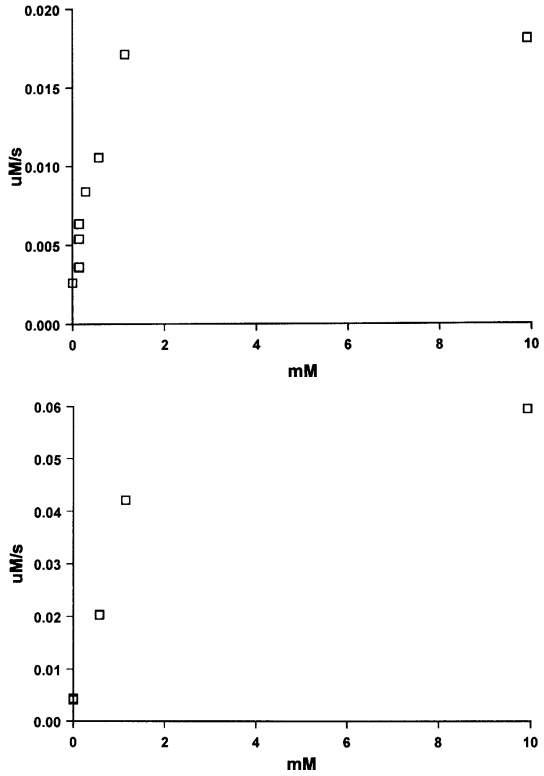


Figure 2 Effect of cellobiose on the rate of liberation of fluoride ions from α-cellobiosyl fluoride by *T. reesei* Cel6A (upper panel) and *H. insolens* Cel6A (lower panel)

The concentrations of α-cellobiosyl fluoride used were 0.192 mM for *T. reesei* Cel6A and 0.143 mM for *H. insolens* Cel6A. Fluoride ion liberation is given in units of μM/s.

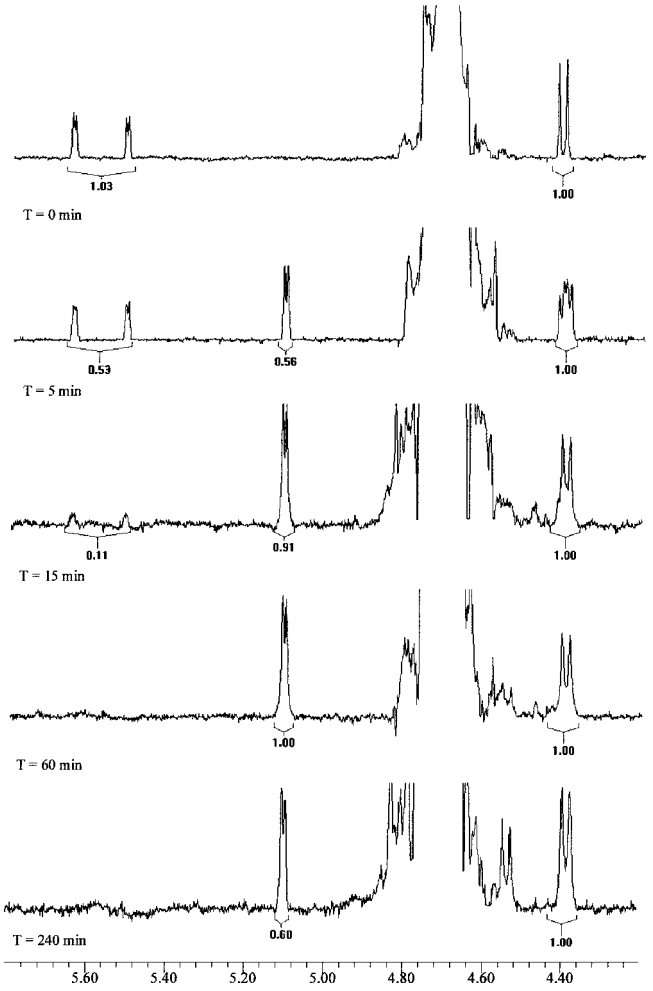


Figure 3 ^{1}H NMR spectra at 400 MHz of the anomeric region of α-cellobiosyl fluoride during the course of its hydrolysis by *H. insolens* CBHII

then inhibition will be observed. At these concentrations, even if cellobiosyl fluoride has the higher affinity for the $-2,-1$ sites, activation by cellobiose will be observed only if cellobiose has a higher affinity for the $+1,+2$ sites than α-cellobiosyl fluoride, and/or the ternary complex of enzyme, α-cellobiosyl fluoride and cellobiose has a higher reactivity than the complex of enzyme with two cellobiosyl fluoride molecules.

In fact, the data in Figure 2 indicate that, for both enzymes, cellobiose indeed activates at low α-cellobiosyl fluoride concentrations. It also activates at high substrate concentrations: at 11.4 mM α-cellobiosyl fluoride the *T. reesei* enzyme is activated 2-fold by 11.4 mM cellobiose, and at 4.8 mM α-cellobiosyl fluoride the *H. insolens* enzyme is activated 4-fold by 7.1 mM cellobiose. This indicates that, for both enzymes, cellobiose has a higher affinity than α-cellobiosyl fluoride for the $+1,+2$ sites, and the mixed ternary complex has a higher reactivity than the complex of enzyme with two α-cellobiosyl fluoride molecules, since activation is observed at both high and low α-cellobiosyl fluoride concentrations.

At this distance in time (and space) it is not possible to identify with confidence the origin of the erroneous kinetic data of Konstantinidis et al. [15]. However, the fact that, without hydrolysis of residual β-cellobiosyl fluoride by CBHI, apparent inhibition of α-cellobiosyl fluoride hydrolysis by *T. reesei* CBHII was observed (results not shown) is suggestive. The logarithmic response of the fluoride electrode makes it possible to measure initial rates at very low conversions, and it is likely that Konstantinidis et al. [15] were in fact measuring the hydrolysis of contaminating β anomer in their freshly prepared samples of α-cellobiosyl fluoride. The material used in the examination of the action of *C. fimi* CenA was 'up to 20% hydrolysed' [13]. Given the approximately 40-fold faster spontaneous hydrolysis of β-glucosyl fluoride compared with that of its anomer [19], spontaneous hydrolysis could well have had the same consequences as our selective hydrolysis with CBHI.

The ^{1}H NMR spectra obtained during the course of hydrolysis of α-cellobiosyl fluoride by *T. reesei* Cel6A were similar to those published previously [15] (which did, however, have a chemical shift scale 0.1 p.p.m. too high), and are therefore not displayed here. The similarities of the two sets of data for complete hydrolysis are in line with our hypothesis that the cause of the initial erroneous kinetic data was reliance on very early initial-rate measurements which were corrupted by 2% stereoisomeric impurity.

The analogous experiment with the *H. insolens* enzyme is displayed in Figure 3. It is seen that the decay of the anomeric resonance of the α-cellobiosyl fluoride at 5.6δ (a doublet of doublets, because of geminal ^{1}H–^{19}F as well as vicinal ^{1}H–^{1}H couplings) is associated with the parallel rise of the anomeric proton of α-cellobiose at 5.1δ. The internal anomeric resonance at 4.4δ shows small changes from hydrolysis of the fluoride link, but no increase in intensity relative to the sum of the intensities

of the the anomeric protons of α-cellobiosyl fluoride and α-cellobiose. If α-cellotetraosyl fluoride had built up in solution before being cleaved, there would have been a reduced intensity of the anomeric peak of α-cellobiose and increased intensity of the peaks due to internal anomeric protons at 4.4δ.

Although on the edge of the HOD (HO^2H) peak, the anomeric proton of the β-cellobiose arising from mutarotation of the α-cellobiose can be seen at $4.5_5\delta$ in the later spectra; this mutarotation of course decreases the intensity of the α-cellobiose peak.

The absence of a detectable build-up of cellotetraosyl fluoride for both enzymes means that, bound in sites -2 to $+2$, a cellotetraoside is hydrolysed faster than it is released. The k_{cat}/K_m values for all cello-oligosaccharides with a degree of polymerization of $\geqslant 4$ are roughly constant at 10^6 $M^{-1}\cdot s^{-1}$ [20]; this is about an order of magnitude slower than the diffusion limit for an enzyme–substrate combination. If k_{cat}/K_m values approach the diffusion limit, the substrate is 'sticky' and the off rate is lower than that of the forward transformation of the substrate. The fact that cellotetraosyl fluoride (and presumably cellotetraose itself) is hydrolysed without being released from the enzyme active site therefore suggests that a simple two-step kinetic mechanism for CBHII is too simple, since the k_{cat}/K_m value for cellotetraose is an order of magnitude too low for the substrate to be sticky by this mechanism. The possibility of a kinetically irreversible conformational change after initial binding of the cellotetraose suggests itself. A similar change in the protein could also explain the apparent infinite co-operativity in the binding of α-cellobiosyl fluoride: the binding of the first cellobiosyl fluoride molecule changes the preferred conformation of the protein from one in which the affinity of the second substrate molecule is weak to one in which it is high. In this case, though, with the poorer substrate, the conformational change would be fast.

M. L. S. apologizes for the misleading nature of the kinetic results reported in Konstantinidis et al. [15].

Received 23 June 1999/14 October 1999; accepted 5 November 1999

Financial support from the European Union Biotechnology Programme (Project BIO4-CT96-0580) and a contribution to the purchase of a 400 MHz NMR spectrometer at UMIST from EPSRC grant GR/L52246 are gratefully acknowledged.

REFERENCES

1 Koshland, D. E. (1953) Biol. Rev. **28**, 416–436
2 Davies, G., Sinnott, M. L. and Withers, G. (1998) in Comprehensive Biological Catalysis, vol. I (Sinnott, M. L., ed.), pp. 119–208, Academic Press, London
3 Davies, G. and Henrissat, B. (1995) Structure **3**, 853–859
4 Hehre, E. J., Brewer, C. F. and Genghof, D. S. (1979) J. Biol. Chem. **254**, 5942–5950
5 Hehre, E. J. (1995) ACS Symp. **618**, 68–78
6 Henrissat, B. and Bairoch, A. (1996) Biochem. J. **316**, 695–696
7 Cottaz, S., Henrissat, B. and Driguez, H. (1996) Biochemistry **35**, 15256–15259
8 Spezio, M., Wilson, D. B. and Karplus, P. A. (1993) Biochemistry **32**, 9906–9916
9 Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J. K. C. and Jones, T. A. (1990) Science **249**, 380–386
10 Varrot, A., Hastrup, S., Schülein, M. and Davies, G. J. (1999) Biochem. J. **337**, 297–304
11 Henrissat, B. and Davies, G. J. (1997) Curr. Opin. Struct. Biol. **7**, 637–644
12 Zou, J., Kleywegt, G. J., Ståhlberg, J., Driguez, H., Nerinckx, W., Claeyssens, M., Koivula, A., Teeri, T. T. and Jones, T. A. (1999) Structure **7**, 1035–1045
13 Damude, H. G., Ferro, V., Withers, S. G. and Warren, R. A. J. (1996) Biochem. J. **315**, 467–472
14 Damude, H. G., Withers, S. G., Kilburn, D. G., Miller, R. C. and Warren, R. A. J. (1995) Biochemistry **34**, 2220–2224
15 Konstantinidis, A., Marsden, I. and Sinnott, M. L. (1993) Biochem. J. **291**, 833–838
16 Hayashi, M., Hashimoto, S. I. and Noyori, R. (1984) Chem. Lett. 1747–1750
17 Szarek, W. A., Grynkiewicz, G., Doboszewski, B. and Hay, G. W. (1984) Chem. Lett. 1751–1754
18 Schülein, M., Tikhomirov, D. F. and Schou, C. (1993) in *Trichoderma reesei* Cellulases and Other Hydrolases (Suominen, P. and Reinikainen, T., eds.), pp. 109–116, Akateeminen Kirjakauppa, Helsinki
19 Zhang, Y., Bommuswamy, J. and Sinnott, M. L. (1994) J. Am. Chem. Soc. **116**, 7557–7563
20 Koivula, A., Kinnari, T., Harjunpää, V., Ruohonen, L., Teleman, A., Drakenberg, T., Rouvinen, J., Jones, T. A. and Teeri, T. T. (1998) FEBS Lett. **429**, 341–346