Hydrolyses of α - and β -cellobiosyl fluorides by cellobiohydrolases of *Trichoderma reesei*

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Cellobiohydrolase II hydrolyses α - and β -D-cellobiosyl fluorides to α -cellobiose at comparable rates, according to Michaelis–Menten kinetics. The stereochemistry, absence of transfer products and strict hyperbolic kinetics of the hydrolysis of α -cellobiosyl fluoride suggest that the mechanism for the α -fluoride may be the enzymic counterpart of the S_N1 reaction observed in the trifluoroethanolysis of α -glucopyranosyl fluoride [Sinnott and Jencks (1980) J. Am. Chem. Soc. 102, 2026–2032]. The

absolute factors by which this enzyme accelerates fluoride ion release are small and greater for the α -fluoride than for the β , suggesting that its biological function may not be just glycoside hydrolysis. Cellobiohydrolase I hydrolyses only β -cellobiosyl fluoride, which is, however, an approx. 1–3% contaminant in α -cellobiosyl fluoride as prepared and purified by conventional methods. Instrumental assays for the various components of the cellulase complex are discussed.

INTRODUCTION

The cellulase complex of the Ascomycete *Trichoderma reesei* is of major biotechnological importance, and consists of at least five discrete soluble proteins, a β -glucosidase, two endoglucanases and two apparently exo-acting cellobioside hydrolases, CBHI and CBHII [1]. The sequences of the genes coding for CBHI [2,3] and CBHII [4] have been determined. These sequences and partial proteolysis studies [5,6] reveal that both enzymes have three domains: a cellulose-binding region, a heavily glycosylated linkage region and a catalytic domain, the cellulose-binding domain being at the C-terminus of CBHI and the N-terminus of CBHII. The cellulose-binding domains are strongly homologous, but there is no homology between the catalytic domains. N.m.r. studies of the isolated cellulose-binding domain of CBHI [7] show that it is wedge-shaped. The tertiary structure of the CBHII catalytic domain, as determined by X-ray crystallography [8], reveals a tunnel for the glucan chain of the substrate and a roughly orthogonal tunnel of water molecules.

When acting on β -cellobiosyl derivatives (including the fluorides) [9], CBHII yields α -cellobiose and CBHI yields β -cellobiose [9,10]. These cellobiohydrolases present the puzzle that absolute k_{cat} values on specific soluble substrates are very low (approx. $10^{-1} s^{-1}$) [11,12], whereas k_{cat} values for glycosidases with biological function that is unquestionably glycoside hydrolysis have k_{cat} values of the order of 10^2 – $10^3 s^{-1}$ for specific substrates [13]. Moreover, work on the experimental evolution of another glycosidase, the *ebg* enzyme of *Escherichia coli*, has shown that unambiguous application of selection pressure to the catalytic efficiency of this enzyme can produce changes in kinetic parameters of one order of magnitude as a consequence of a single mutational event [14–16]. It therefore seems unlikely that the genes coding for CBHI and CBHII are under any selection pressure to increase the catalytic efficiency of their hydrolysis of β -glucan linkages *per se*. We now present data on the hydrolysis of cellobiosyl fluorides by CBHII which indicate that the molecular machinery of this enzyme is so little directed towards cleavage of the β -cellobiosyl linkage that, with a small leaving

group, it accelerates cleavage of an α -cellobiosyl linkage rather more.

We also present kinetic data on hydrolysis of cellobiosyl derivatives by CBHI, which show that the k_{cat} value for β -cellobiosyl fluoride is about two orders of magnitude greater than that for aryl cellobiosides, even of acidic aglycones. The hydrolysis of the cellobiosyl–enzyme intermediate is unlikely therefore to govern k_{cat} for the aryl cellobiosides.

MATERIALS AND METHODS

Hepta-*O*-acetyl- α - and - β -cellobiosyl fluorides (m.p. 187 °C and 165 °C respectively) and hepta-*O*-acetyl α -lactosyl fluoride (m.p. 82 °C) were made by published procedures [17], although some of the α -cellobiosyl fluoride was also made by the action of HF/pyridine [18,19] on octa-acetylcellobiose. Particular attention was paid to the anomeric purity of this fluoride, which was examined by 1H and ^{19}F n.m.r. (1H δ 5.65 p.p.m., dd, J_{HF} 53 Hz, J_{HH} 2.9 Hz). (We showed that 5% of the hepta-acetyl β -fluoride as an impurity in its α -anomer could readily be detected.) The acetylated disaccharide fluorides were deacetylated immediately before use [20] using sodium acetate (3 ml of 0.5% in methanol) in dry methanol (10 ml) and examined by ^{19}F n.m.r.

3,4-Dinitrophenyl cellobioside {m.p. 205–207 °C (dec), $[\alpha]_D^{25}$ –7.8° (water)} was made by the method of Capon and Thomson [21]. 3,4-Dinitrophenyl lactoside {m.p. 223–224 °C (dec), $[\alpha]_D^{25}$ –69.5° (water) (Found: C, 42.70; H, 4.84; N, 5.01%; $C_{18}H_{24}N_2O_{15}$ requires C, 42.62; H, 4.72; N, 5.51%)} was made similarly.

T. reesei QM9414 or Rut-C-30 was grown on cellulose as described by Mandels and Weber [22]. After 4–7 days the mycelia and cellulose were centrifuged off (20 min, 11 000 *g*), phenylmethanesulphonyl fluoride (about 1 mM) was added, the culture filtrate concentrated in an Amicon PM10 concentrator using polysulphonate membranes, dialysed with Spectra-Por CE dialysis tubes and fractionated on a Protein PAK-Glass-DEAE 5PW column on a Waters 650 protein-isolation system (flow rate 1 ml/min, ammonium acetate 5–250 mM as eluent). Fractions

containing CBHI, detected by its ability to hydrolyse 3,4-dinitrophenyl lactoside, and CBHII, detected by its ability to hydrolyse α -cellobiosyl fluoride, were further purified on an affinity column of *p*-aminobenzyl 1-thio- β -cellobioside–Sepharose [23]. The isolated enzymes gave one band on SDS/PAGE (Pharmacia PHAST system), and were kinetically and electrophoretically indistinguishable from enzymes kindly provided earlier [9] by Dr. Päivi Lehtovaara and Dr. Tuula Teeri (VTT Biotechnology Laboratory, Espoo, Finland); initial characterization of the hydrolysis of α -cellobiosyl fluoride by CBHII was made with enzyme from this source.

The fluoride-electrode-based system for following the hydrolysis of glycosyl fluorides has been described elsewhere [24].

The hydrolysis of α -cellobiosyl fluoride by CBHII was followed at 400 MHz on a Bruker AM 400 spectrometer using a dual $^{13}\text{C}/^1\text{H}$ probe. Spectra were collected in 32K data points and zero filled to 64K before Fourier transformation. The enzyme (0.48 mg/ml) was exchanged with sodium acetate/acetic acid buffer in $^2\text{H}_2\text{O}$ (pD 4.6) five times. Because of the proximity of the HO^2H peak to the resonances of interest, electronic integration is unreliable, and peak areas were estimated by expansion and cutting and weighing.

The hydrolysis of 3,4-dinitrophenyl glycosides were monitored continuously at 390 nm ($\Delta\epsilon$ 4426 $\text{M}^{-1}\cdot\text{cm}^{-1}$), and the hydrolyses of *p*-nitrophenyl and 4-methylumbelliferyl cellobiosides were followed by stopping the reaction with 1 M NaOH and measuring the absorbance of *p*-nitrophenolate at 402 nm ($\Delta\epsilon$ 18000 $\text{M}^{-1}\cdot\text{cm}^{-1}$) or the fluorescence of 4-methylumbelliferone anion at 435 nm (from excitation at 335 nm).

RESULTS AND DISCUSSION

Table 1 gives k_{cat} and K_{m} values for the hydrolyses of disaccharide derivatives by CBHI and CBHII, k_{cat} values being calculated on the assumption that the enzymes as first isolated were 100% active protein, and that the M_r of CBHI was 65000 [5] and that of CBHII was 58000 [4]. Data refer to 0.1 M sodium acetate/acetic acid buffer, pH 5, at 25 °C.

Hydrolyses by CBHII

It has long been known [25,26] that glycosidases hydrolyse the corresponding glycosyl fluoride, but the k_{cat} value for the β -fluoride is still remarkable because of its low absolute values.

It has also been known for some time that inverting glycosidases hydrolyse glycosyl fluorides of both anomeric configurations (e.g. [27,28] reviewed in [13]). However, the hydrolysis of α -cellobiosyl fluoride by CBHII appears to take place by a novel mechanism. The general mechanism of hydrolysis of normal substrates by inverting glycosidases such as CBHII is thought to be a single development, involving proton donation to the leaving group by an acid-catalytic group A-H and deprotonation of a nucleophilic water molecule by a base B (Scheme 1).

The characteristics of those hydrolyses of the 'wrong' fluoride by inverting glycosidases that have hitherto been examined have been consistent with a resynthesis–hydrolysis mechanism, due to E. J. Hehre, in which a saccharide linkage is first synthesized by the action of a tautomer of the usually catalytically active form of the enzyme on two fluoride molecules. This is illustrated for the hydrolysis of α -xylosyl fluoride by the inverting β -xylosidase of *Bacillus pumilus* (Scheme 2) [27]. In this mechanism, proton donation to the leaving fluoride by BH^+ is combined with

abstraction of a proton from the hydroxy group of a second molecule of glycosyl fluoride by A^- , to synthesize a new saccharide linkage of the 'right' anomeric configuration, which is then hydrolysed by the usual mechanism. Thus the hydrolysis of α -xylosyl fluoride by the inverting β -xylosidase of *B. pumilus* involves the initial synthesis of α -xylobiosyl fluoride, and likewise the hydrolysis of β -maltosyl fluoride by β -amylase involves the initial production of β -maltotetraosyl fluoride, which is then hydrolysed to β -maltose and β -maltosyl fluoride [28]. The Hehre mechanism requires that, at low enough concentrations of glycosyl fluoride, the rate of fluoride ion production should be proportional to the square of the glycosyl fluoride concentration:

Table 1 Michaelis–Menten parameters for hydrolysis of disaccharide derivatives by CBHI and CBHII of *T. reesei* in 0.1 M sodium acetate/acetic acid buffer, pH 5, at 25 °C

	k_{cat} (s^{-1})	K_{m} (mM)
CBHI		
β -Cellobiosyl fluoride	0.61	0.31
3,4-Dinitrophenyl lactoside	0.65	0.38
4-Nitrophenyl cellobioside*	0.0029	0.064
4-Methylumbelliferyl cellobioside†	0.0044	0.051
3,4-Dinitrophenyl cellobioside	0.0074	0.29
CBHII		
α -Cellobiosyl fluoride	0.50	0.52
β -Cellobiosyl fluoride	4.0	0.15
Cellobiose (K_i)		2.6

* Woodward et al. [39] give $K_{\text{m}} = 3.4$ mM for a CBHI preparation not subjected to affinity chromatography.

† van Tilbeurgh et al. [40] give $k_{\text{cat}} = 0.002$ s^{-1} and $K_{\text{m}} = (3.5 \pm 0.8) \times 10^{-5}$ M.

Scheme 1 Conventional single-displacement mechanism for an inverting β -glycosidase ($e \rightarrow a$ glycosidase), shown for a β -xylosidase

Scheme 2 First step in the hydrolysis of α -xylosyl fluoride by the inverting β -xylosidase of *Bacillus pumilus*

Figure 1 Rate of liberation of fluoride ion from α -cellobiosyl fluoride by CBHII ($0.56 \mu\text{M}$) in 0.1 M sodium acetate/acetic acid buffer at 25°C , as a function of substrate concentration

The inset shows data at low substrate concentration

Figure 2 Double-reciprocal plot of the liberation of fluoride ion from α -cellobiosyl fluoride by CBHII ($0.56 \mu\text{M}$) in 0.1 M sodium acetate/acetic acid buffer at 25°C in the absence (●) and presence (▲) of fully mutarotated cellobiose (0.012 M)

The lines describe values of V_{max} and K_m calculated from both sets of data by non-linear least-squares fitting of the experimental points directly to the Michaelis–Menten equation, using the program KALEIDAGRAPH. (The enzyme stock solution has been stored for 6 months at 4°C after the Figure 1 experiment had been performed).

further, it is often possible to replace the second molecule of glycosyl fluoride with another saccharide and observe stimulation of fluoride ion production, and also to observe the formation of transfer products directly.

From Figure 1, however, it is clear that the hydrolysis of α -cellobiosyl fluoride obeys strictly Michaelian kinetics to the limits of our instrumentation: there is no sign of a greater than first-order dependence of rate on α -cellobiosyl fluoride concentration. Moreover, Figure 2 indicates that cellobiose acts as a competitive inhibitor, whereas, if the Hehre mechanism had been operating, one would expect to see a stimulation of fluoride ion production, at least at low cellobiosyl fluoride concentration, as the cellobiose replaced the second molecule of cellobiosyl fluoride. There is therefore no kinetic evidence for a resynthesis–hydrolysis mechanism for hydrolysis of this compound.

If the reaction is examined by n.m.r., only the direct conversion of α -cellobiosyl fluoride into α -cellobiose can be detected. Figure 3 displays n.m.r. spectra of the anomeric region of a solution of α -cellobiosyl fluoride during the course of its hydrolysis by CBHII. Peak intensities are shown as a function of time in Figure 4. It is clear that production of α -cellobiose parallels loss of substrate and that no intermediate product can be detected.

CBHII therefore appears not to hydrolyse α -cellobiosyl fluoride by the Hehre mechanism. This is in accord with the X-ray-crystal structure of the catalytic domain of the protein [8], which reveals no plausible candidate for the base B, merely a tunnel of water molecules going from the outside of the protein to the fissile saccharide residue. By contrast, the crystal structure of *Aspergillus* glucoamylase, an inverting glycosidase which hydrolyses β -glucosyl fluoride by the Hehre mechanism [29], reveals a clearly defined catalytic base (Glu-400) hydrogen-bonded to a water molecule correctly placed on the β face of the saccharide residue to act as a nucleophile [43,44]. This enzyme has structural similarities with the inverting endoglucanase (*CelD*) from *Clostridium thermocellum*, in which again a base catalyst (Asp-201) can be discerned [30].

A possible mechanism for the hydrolysis of α -cellobiosyl fluoride by CBHII would be an S_Ni reaction in which a normally nucleophilic water molecule acts as a hydrogen-bond donor to the departing fluoride ion: the resulting ion pair of glycosyl cation and $\text{F}^- \dots \text{HOH}$ then recombines on oxygen, resulting in overall retention of the anomeric configuration. A process closely resembling this has been observed in the trifluoroethanolysis of α -glucosyl fluoride (which yields largely trifluoroethyl α -glucoside [31]).

The spontaneous hydrolysis rate of α - [24,32] and β - [24] glucosyl fluoride has been measured. Extrapolation of these data to 25°C gives first-order rate constants for uncatalysed loss of fluoride ion for α - and β -glucosyl fluorides of $2.3 \times 10^{-7} \text{ s}^{-1}$ and $3.6 \times 10^{-5} \text{ s}^{-1}$ respectively. On the assumption that the remote β -glucopyranosyl residue on the reactive saccharide unit of a disaccharide fluoride makes little difference to the non-enzymic rate, the absolute rate enhancement brought about by CBHII on the hydrolysis of α -cellobiosyl fluoride is seen to be 2.2×10^6 and for β -cellobiosyl fluoride is 1.1×10^5 , if it is further assumed that, for this single-displacement enzyme, C–F bond cleavage limits k_{cat} . These factors are small: rate enhancements of 10^{10} – 10^{12} are commonly observed for glycosidase-catalysed hydrolyses of *O*- and *N*-glycosides [13]. Analysis of the kinetics of hydrolysis of small substrates by glycosidases which normally act on extended oligosaccharides must take into account the possibilities of both non-productive binding (which will produce an apparently low k_{cat}) and a requirement for occupancy of all the saccharide-binding sites for complete recruitment of catalytic power. The former possibility is unlikely since the X-ray studies have revealed that the proximal saccharide residue of iodobenzyl thiocellobioside binds to the putative cleavage subsite of the catalytic domain of CBHII [8], but the latter may account for some of the catalytic feebleness towards disaccharide substrates: it has recently been found that cellotetraose has a k_{cat} of 3.3 s^{-1} compared with $3 \times 10^{-2} \text{ s}^{-1}$ for cellotriose (A. Teleman and T. Teeri, personal communication). However, the precedent of glucoamylase, which achieves a rate enhancement of $10^{7.9}$ on α -glucosyl fluoride despite normally acting on malto-oligosaccharides [24], suggests that the requirement for an extended substrate is not the only reason for low catalytic power. Such a requirement, moreover, has no bearing on the rate enhancement being 20-fold bigger for the ‘wrong’ (α) glycosyl fluoride than the ‘right’ (β) one. This stereochemical indiscrimination is the strongest circumstantial evidence that glycoside hydrolysis itself

Figure 3 ^1H n.m.r. spectra at 400 MHz of the anomeric region of α -cellobiosyl fluoride during the course of its hydrolysis by CBHII

For details see the Materials and methods section. The doublet of doublets centred on δ 5.70 corresponds to the anomeric proton of α -cellobiosyl fluoride, split by the proton on C2 ($J_{1,2}$ 2.57 Hz) and the fluorine ($^2J_{\text{HF}}$ 53.35 Hz). The anomeric proton of α -cellobiose appears at δ 5.23 ($J_{1,2}$ 3.85 Hz) and of β -cellobiose at δ 4.67 ($J_{1,2}$ 7.95 Hz) (just on the side of the large HO^2H peak). Changes in the anomeric proton of the internal saccharide linkage ($J_{4,5}$ 7.95 Hz), from δ 4.55 in the fluoride to δ 4.52 in cellobiose, can also be detected. The vicinal proton–proton couplings establish the stereochemistry at the anomeric centre, the smaller couplings being characteristic of a *cis* arrangement (and hence an α configuration), the larger couplings of a *trans* diaxial arrangement (and hence a β configuration).

is not the primary biological function of the protein, and suggests that the portion of the catalytic machinery of the protein directed towards glycoside hydrolysis is merely a pattern of electrostatic fields that stabilize glycosyl-cation-like transition states, of the type first suggested by Warshel [33] for lysozyme. These fields can thus be considered as stabilizing indiscriminately transition states for direct and $\text{S}_{\text{N}}\text{i}$ displacements of the small fluoride ion leaving group.

It is tempting to speculate that CBHII is in fact part of the ' C_1 ' component of the cellulase complex first proposed by Reese et al. [34], and that it brings about a coupled vectorial process (see e.g. [35]), in which the free energy of hydrolysis of the glycosidic

linkage is coupled to mechanical disruption of the cellulose crystallite.

Coughlan and Ljungdahl [36] used the term 'destratification' to describe disruption of the cellulose crystallite by CBH enzymes, but suggested that such a process could occur without the release of reducing sugars. Knowles et al. [37] suggested that the cellulose-binding domain 'unzipped' the glucan chain from the cellulose crystallite and the catalytic domain hydrolysed the glycosidic bond. The key feature of our present suggestion, though, is that the energetics of the two processes are necessarily coupled, and that the energy for disruption of the cellulose crystallite comes from the hydrolysis of the glucosidic bond (the standard free

Figure 4 Intensities of the anomeric protons of α -cellobiosyl fluoride ($\circ$), α -cellobiose ($\bullet$) and β -cellobiose ($\triangle$) as a function of time

Intensities are expressed as % of a proton. The anomeric equilibrium of cellobiose favours the β -anomer.

energy of hydrolysis of the $\beta(1\rightarrow4)$ linkage in lactose, which will be very similar to that in cellobiose, being -13 kJ/mol (molar standard state) [38]. In our model, the glycosidase activity of CBHII plays much the same role as the ATPase activity of myosin.

Hydrolyses by CBHI

Initial experiments with the (retaining) CBHI indicated that this enzyme too hydrolysed α -cellobiosyl fluoride, by a process that was inhibited by cellobiose with the same K_i as the hydrolysis of conventional substrates ($20\ \mu\text{M}$). However, we were never able to obtain more than (apparently) 7% hydrolysis (corresponding to $8.27\ \mu\text{M F}^-$) of α -cellobiosyl fluoride. Because of the low K_i value of the product cellobiose, with this substrate it was not really possible to distinguish the effects of contamination by the β -fluoride from those of strong product inhibition. With α -lactosyl fluoride, made the same way as α -cellobiosyl fluoride, as substrate, however, an apparent 'burst' of F^- liberation, independent of enzyme concentration, and corresponding to approx. 1% of total substrate concentration, was observed at concentrations of substrate below the K_i for lactose. After the 'burst', no further liberation of fluoride ion was observed. It is therefore clear that the apparent hydrolysis of α -cellobiosyl fluoride by CBHI is an artifact of its stereochemical impurity.

In Table 1 are compared Michaelis–Menten parameters for CBHI-catalysed hydrolyses of β -cellobiosyl fluoride, 4-nitrophenyl cellobioside, 3,4-dinitrophenyl cellobioside, 4-methylumbelliferyl cellobioside and 3,4-dinitrophenyl lactoside. It can be seen that k_{cat} values are low, presenting the same paradox, in the light of the experimental evolution results on *ebg*, as CBHII. However, the value for the β -cellobiosyl fluoride exceeds by a couple of orders of magnitude those for aryl cellobiosides, so that our previous speculation [13], that the very low k_{cat} values for these substrates reflect a low rate of hydrolysis of the cellobiosyl-enzyme intermediate implied by the stereochemistry of the catalysed reaction, is unlikely to be correct. In accord with this, attempts to detect a burst of 3,4-dinitrophenol by stopped-flow techniques, or to detect kinetically the interception of the cellobiosyl–CBHI with added nucleophile, failed.

Instrumental assay of components of the cellulase complex of *T. reesei*

The discovery of Claeysen's group [40] that CBHI, alone amongst the enzymes of the cellulase complex, will hydrolyse aryl lactosides provides the basis for convenient specific instrumental assays of this enzyme. We now report data for the 3,4-dinitrophenyl lactoside. The 3,4-dinitrophenol leaving group, introduced by Capon and Thomson [21], has the advantage over *p*-nitrophenol that it is ionized at the pH optima of the cellulase enzymes, so that continuous spectrophotometric assays can be performed. It also has the advantage over 4-methylumbelliferone that the leaving group fluorescence does not have to be calibrated for each set of assays and the advantage over 2,4-dinitrophenol that the rate of spontaneous hydrolysis is negligible. Claeysen's group introduced the very similar 2-chloro-4-nitrophenol leaving group [41].

In principle, the electrochemical monitoring of the hydrolysis of α -cellobiosyl fluoride should provide for the first time a specific continuous instrumental assay of CBHII. The difficulty of purifying the substrate from its β -anomer, which should be a substrate for all five enzymes of the cellulase complex, means though that the results of such assays should be interpreted with caution. Our ability to detect by ^1H n.m.r. β -hepta-acetyl-cellobiosyl fluoride as a 3% impurity in its α -anomer, and at the same time hydrolyse with CBHI 7% of a sample of α -cellobiosyl fluoride which had been prepared from a sample of its hepta-acetate that was provably less impure, indeed raises the possibility of spontaneous anomerization of the deprotected sugar at some stage. Nonetheless, the assay without rigorously purified substrate is adequate to locate fractions containing CBHII during purification of culture filtrates, but for finer assays it is probably better to take, not initial rates, but rates of hydrolysis of $> K_m$ concentrations of substrate, after approx. 5% hydrolysis.

It is thus possible to have specific instrumental assays for both cellobiohydrolases of the *T. reesei* cellulase complex. It is also possible to envisage a specific spectrophotometric assay of endoglucanase II/III, as only this enzyme will liberate the chromophoric leaving group from aryl cellotriosides [42]. However, unlike the cellobiohydrolase assays, this assay is not readily applicable to crude culture filtrates, as aglycone could be liberated by the sequential action of the cellobiohydrolases and β -glucosidase.

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