# Mechanisms of Cellulases and Xylanases: A Detailed Kinetic Study of the Exo-β-1,4-glycanase from *Cellulomonas fimi*†

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*Received January 13, 1994; Revised Manuscript Received March 21, 1994*®

ABSTRACT: The exoglucanase/xylanase from *Cellulomonas fimi* (Cex) has been subjected to a detailed kinetic investigation with a range of aryl β-D-glycoside substrates. This enzyme hydrolyzes its substrates with net retention of anomeric configuration, and thus it presumably follows a double-displacement mechanism. Values of $k_{cat}$ are found to be invariant with pH whereas $k_{cat}/K_m$ is dependent upon two ionizations of $pK_a$ = 4.1 and 7.7. The substrate preference of the enzyme increases in the order glucosides < cellobiosides < xylobiosides, and kinetic studies with a range of aryl glucosides and cellobiosides have allowed construction of Broensted relationships for these substrate types. A strong dependence of both $k_{cat}$ ($\beta_{lg} = -1$) and $k_{cat}/K_m$ ($\beta_{lg} = -1$) upon leaving group ability is observed for the glucosides, indicating that formation of the intermediate is rate-limiting. For the cellobiosides a biphasic, concave downward plot is seen for $k_{cat}$, indicating a change in rate-determining step across the series. Pre-steady-state kinetic experiments allowed construction of linear Broensted plots of log $k_2$ and log ($k_2/K_d$) for the cellobiosides of modest ($\beta_{lg} = -0.3$) slope. These results are consistent with a double-displacement mechanism in which a glycosyl-enzyme intermediate is formed and hydrolyzed *via* oxocarbonium ion-like transition states. Secondary deuterium kinetic isotope effects and inactivation experiments provide further insight into transition-state structures and, in concert with $\beta_{lg}$ values, reveal that the presence of the distal sugar moiety in cellobiosides results in a less highly charged transition state. These studies suggest that the primary function of the distal sugar is to increase the rate of formation of the glycosyl-enzyme intermediate through improved acid catalysis and greater nucleophile preassociation, without affecting its rate of decomposition.

Cellulases have been the focus of considerable recent attention as a result of their potential in biomass degradation and fuel production. They have been isolated from a variety of organisms, and they have been cloned, sequenced, and divided into several families on the basis of sequence similarity (Beguin, 1990; Gilkes et al., 1991; Henrissat, 1993). Many of these enzymes are composed of two distinct domains, a catalytic domain and a cellulose-binding domain. Information on their three-dimensional structure is, however, somewhat limited. Detailed three-dimensional X-ray crystallographic structures have been published for the catalytic domains of cellobiohydrolase II from *Trichoderma reesei* (Rouvinen et al., 1990), endoglucanase D from *Clostridium thermocellum* (Juy et al., 1992), thermophilic endocellulase $E_2$ from *Thermomonospora fusca* (Spezio et al., 1993), and endoglucanase V from *Humicola insolens* (Davies et al., 1993). In addition, the structure of the cellulose-binding domain from the *Trichoderma* enzyme has been determined by $^1$H-NMR (Kraulis et al., 1989), and low-angle X-ray studies on endoglucanase A (CEN A) and exoglycanase (Cex)[1] from *Cellulomonas fimi* have revealed a tadpole-like structure for the intact enzymes (Pilz et al., 1990). Other structural information available has been derived by labeling of these enzymes with mechanism-based inactivators (Tull et al., 1991; Wang et al., 1993) and affinity labels (Hoj et al., 1989, 1992).

Other studies performed on cellulases have included specificity mapping with oligosaccharides in order to determine preferred sites of cleavage (Claeyssens & Henrissat, 1992; Bhat et al., 1990; van Tilbeurgh et al., 1985), determination of cleavage stereochemistry (Withers et al., 1986; Gebler et al., 1992b), and a number of mutagenesis studies. These have largely involved the mutation of conserved residues followed by assay of residual activity. Such studies have provided valuable insights into the identities of residues involved in catalysis.

Kinetic studies performed on cellulase systems have been limited, largely because of the problems associated with kinetic studies on crystalline or modified celluloses, and the difficulties involved in the synthesis of suitably modified soluble substrates. However, such studies are extremely useful as they allow delineation of the mechanism in some detail through identification of rate-limiting steps, and then further investigation of the transition-state structure for each step. Careful studies of this type have been performed on several monosaccharide hydrolases such as *Escherichia coli* β-galactosidase and *Agrobacterium faecalis* β-glucosidase, and these have involved establishment of Broensted relationships and measurement of primary and secondary kinetic isotope effects as well as pre-steady-state kinetics [see review by Sinnott (1990) and Kempton and Withers (1992)]. Very few such studies have been performed on a cellulase, and in no case has a comprehensive study involving all these techniques been carried out in a single enzyme of this type.

The gene encoding the exoglycanase from *C. fimi* (Cex) has been cloned, expressed in *E. coli,* and subsequently

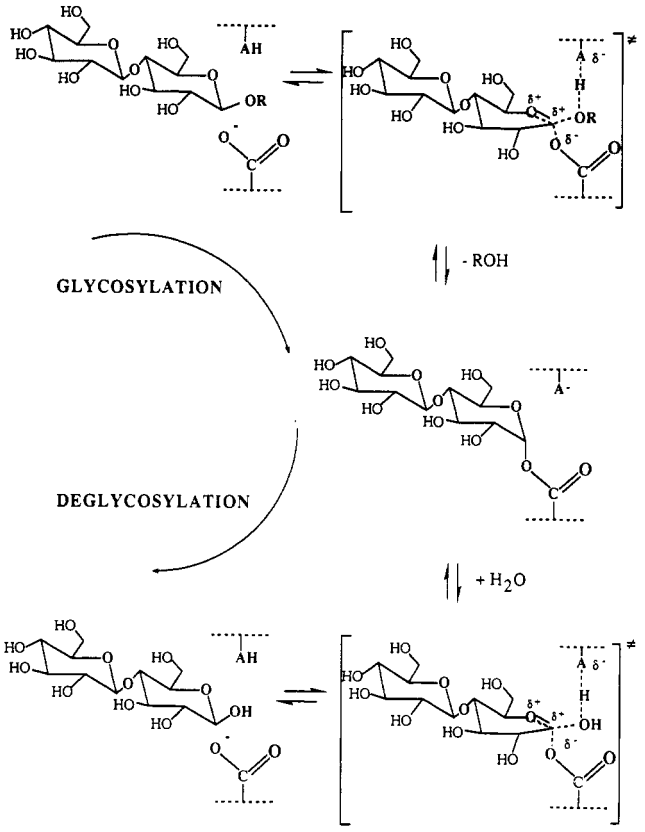
† This work was supported by the British Columbia Science Council, by the Natural Sciences and Engineering Research Council of Canada, and by the Protein Engineering Network of Centres of Excellence, one of the fifteen Networks of Centres of Excellence supported by the Government of Canada.

® Abstract published in *Advance ACS Abstracts,* May 1, 1994.

[1] Abbreviations: Cex, *Cellulomonas fimi* exoglycanase; 2,4-DNPC, 2″,4″-dinitrophenyl β-cellobioside; 4-BrPC, 4″-bromophenyl β-cellobioside; 2,4-DNPG, 2′,4′-dinitrophenyl β-D-glucopyranoside; 2F-DNPG, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside; 2F-DNPC, 2″,4″-dinitrophenyl 2-deoxy-2-fluoro-β-cellobioside; BSA, bovine serum albumin; $ONPX_2$, 2″-nitrophenyl xylobioside; $PNPX_2$, 4″-nitrophenyl xylobioside; PNPG, 4′-nitrophenyl β-D-glucopyranoside; PNPC, 4″-nitrophenyl β-cellobioside.

0006-2960/94/0433-6363$04.50/0 

Scheme 1: Proposed Mechanism of Action of *C. fimi* Exoglycanase




sequenced (O'Neill et al., 1986). This enzyme is a 47-*k*Da protein comprising two domains which are separable by limited proteolysis: an active catalytic domain (35 *k*Da) and a cellulose-binding domain (~12 *k*Da) which only binds cellulose (Gilkes et al., 1988). The catalytic domain has been crystallized, yielding crystals which diffract to better than 2.0-Å resolution (Bedarkar et al., 1992), but the structure is as yet unsolved. Previous investigations of Cex revealed it to be active on cellulose, xylan, and small substrates such as aryl β-xylobiosides, aryl β-cellobiosides, and aryl β-D-glucopyranosides (Gilkes et al., 1984; Tull et al., 1991). A sterochemical study of this exoglycanase using $^1$H-NMR showed it to be a "retaining" glycosidase; thus a double-displacement mechanism in which a glycosyl-enzyme intermediate is formed and hydrolyzed with acid/base catalytic assistance *via* oxocarbonium ion-like transition states has been proposed (Withers et al., 1986) (Scheme 1). Such a mechanism, originally proposed by Koshland (1953), is considerably more likely than alternatives involving ring opening (Post & Karplus, 1986) which lack serious experimental support. This paper describes the detailed mechanistic investigation of this enzyme, the first such study on a cellulase.

## MATERIALS AND METHODS

*Materials.* 4′-Nitrophenyl and 2′-nitrophenyl β-D-glucopyranosides, 4″-nitrophenyl and 2″-nitrophenyl β-cellobiosides, and all buffer chemicals were obtained from Sigma Chemical Co. Phenols were obtained from either Sigma Chemical Co., Aldrich Chemical Co., or Fluka. 2′-Chloro-4′-nitrophenyl β-D-glucopyranoside was a generous gift from Dr. Marc Claeyssens. Both 4″-nitrophenyl and 2″-nitrophenyl β-xylobioside were synthesized in this laboratory, and their syntheses will be published shortly. Syntheses of 2′,4′-dinitrophenyl, 2′,5′-dinitrophenyl, 3′,4′-dinitrophenyl, and 3′-nitrophenyl β-D-glucopyranosides have been published (Kempton & Withers, 1992). Syntheses of 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside and its cellobioside analogue have been described elsewhere (Withers et al., 1990; McCarter et al., 1993). The exoglycanase was provided by Dr. Neil Gilkes, Dr. Douglas Kilburn, and Dr. Antony Warren, and its purification has been previously described (Gilkes et al., 1984)

*Synthesis.* Aryl 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-β-cellobiosides were synthesized according to the following procedure. Hepta-*O*-acetyl-α-cellobiosyl bromide (Fischer & Zemplen, 1910) dissolved in acetone (~0.4 mmol of acetobromocellobiose/mL) was added to the appropriate substituted phenol dissolved in 1 M NaOH (1 mmol of phenol/mL of base) and stirred at room temperature for 24–48 h. The solvent was then evaporated *in vacuo* and the remaining syrup diluted with water and extracted with chloroform. The organic phase was washed (3 × 50 mL) with saturated sodium bicarbonate, dried over $MgSO_4$, filtered, and evaporated *in vacuo*. The products were crystallized from ethyl acetate/ethanol and fully characterized by $^1$H-NMR and elemental analysis. Deacetylation of the reactive cellobiosides (leaving group p*K*a < 6) was achieved using acetyl chloride in methanol (Ballardie et al., 1973) while the more stable cellobiosides were deprotected using sodium methoxide in methanol (Sinnott & Souchard, 1973). Characterization data for the aryl β-cellobiosides are the following.

*3″,4″-Dinitrophenyl β-cellobioside* was recrystallized from methanol and diethyl ether to yield a pale yellow solid (20 mg, 0.04 mmol, 40%): mp 198–199 °C (lit. mp 187–192 °C) (Capon & Thompson, 1979); $^1$H-NMR ($CD_3OD$): δ 8.15 (d, $J_{5'',6''}$ 11 Hz, H(5″)), 7.65 (d, $J_{2'',6''}$ 2 Hz, H(2″)), 7.47 (dd, $J_{2'',6''}$ 2 Hz, $J_{6''5''}$ 11 Hz, H(6″)), 5.20 (d, $J_{1,2}$ 9 Hz, H(1)), 3.20–5.20 (m, H(2–6) and H(1′–6′)). Anal. Calcd for $C_{18}H_{24}N_2O_{15}$: C, 42.50; H, 4.72; N, 5.51. Found: C, 42.27; H, 4.91; N, 5.42.

*3″,5″-Dichlorophenyl β-cellobioside* was recrystallized from methanol, diethyl ether, and hexane as a white solid (33 mg, 0.067 mmol, 33%): mp 250–253 °C; $^1$H-NMR ($CD_3OD$): δ 7.20 (m, H(4″)), 7.10 (d, *J* 2 Hz, H(2″,6″)), 5.11, (d, $J_{1,2}$ 10 Hz, H(1)), 3.27–4.97 (m, H(2–6) and H(1′–6′)). Anal. Calcd for $C_{18}H_{24}O_{11}C_{12}$: C, 44.35; H, 4.92. Found: C, 44.18; H, 5.08.

*3″-Nitrophenyl β-cellobioside* was recrystallized from methanol, diethyl ether, and hexane to give a white solid (17 mg, 0.037 mmol, 10%): mp 220–222 °C; $^1$H-NMR ($D_2O$): δ 8.00 (m, H(2″,4″)), 7.55 (m, H(5″,6″)), 5.27 (d, $J_{1,2}$ 9 Hz, H(1)), 3.30–5.00 (m, H(2–6) and H(1′–6′)). Anal. Calcd for $C_{18}H_{25}O_{13}N$: C, 46.60; H, 5.39; N, 3.02. Found: C, 46.19; H, 5.47; N, 2.89.

*4″-Cyanophenyl β-cellobioside* was recrystallized as a white powder from methanol and diethyl ether (70 mg, 0.16 mmol, 59%): mp 241–242 °C; $^1$H-NMR ($CD_3OD$): δ 7.70 (d, *J* 9 Hz, H(3″,5″)), 7.23 (d, *J* 9 Hz, H(2″,6″)), 5.12 (d, $J_{1,2}$ 9 Hz, H(1)), 3.20–5.05 (m, H(2–6) and H(1′–6′)). Anal. Calcd for $C_{19}H_{25}O_{11}N{\cdot}0.5H_2O$: C, 50.44; H, 5.75; N, 3.09. Found: C, 50.60; H, 5.96; N, 2.97.

*4″-Bromophenyl β-cellobioside* was recrystallized from methanol and diethyl ether as a white powder (13 mg, 0.026 mmol, 12%): mp 223–224 °C; $^1$H-NMR ($CD_3OD$): δ 7.40 (d, *J* 8 Hz, H(3″,5″)), 7.00 (d, *J* 8 Hz, H(2″,6″)), 4.95 (d, $J_{1,2}$ 8 Hz, H(1)), 3.20–4.90 (m, H(2–6)and H(1′–6′)). Anal. Calcd for $C_{18}H_{25}O_{11}Br$: C, 43.46; H, 5.23. Found: C,43.06; H, 5.09.

*2″,4″-Dinitrophenyl β-cellobioside* was prepared as follows. 2,3,6,2′,3′,4′,6′-Hepta-*O*-acetyl-D-cellobiose (Excoffier et al., 1975) (1.08 g, 1.70 mmol) and 1,4-diazabicyclo[2.2.2]octane (660 mg, 5.90 mmol) were stirred over molecular sieves (4 Å) in DMF (20 mL) for 3 h. Fluorodinitrobenzene (409 mg, 2.20 mmol) was added, and the reaction was allowed to proceed at room temperature for 24 h. The sieves were removed by gravity filtration and washed with chloroform and the combined extracts evaporated *in vacuo*. The remaining yellow solid was dissolved in chloroform (40 mL), washed (3 × 50 mL) with saturated sodium bicarbonate, and dried over anhydrous $MgSO_4$. The solvent was evaporated *in vacuo*, yielding 2″,4″-dinitrophenyl 2,3,6,2′,3′,4′,6′ hepta-*O*-acetyl-β-cellobioside, which was crystallized and recrystallized from ethyl acetate/low boiling petroleum ether and was characterized by $^1$H NMR and elemental analysis. Deacetylation of the cellobioside using acetyl chloride in methanol and recrystallization from methanol and diethyl ether yield the product as a white powder (128 mg, 0.16 mmol, 27%): mp 180 °C dec; $^1$H-NMR ($CD_3OD$): δ 8.72 (s, H(3″)), 8.45 (d, $J_{5'',6''}$ 10 Hz, H(5″)), 7.62 (d, $J_{6'',5''}$ 10 Hz, H(6″)), 5.30 (d, $J_{1,2}$ 6 Hz, H(1)), 3.22–5.00 (m, H(2–6) and H(1′–6′)). Anal. Calcd for $C_{18}H_{24}N_2O_{15}$: C, 42.50; H, 4.72; N, 5.51. Found: C, 42.20; H, 4.87; N, 5.33.

*2″,5″-Dinitrophenyl β-cellobioside* was synthesized as follows. Hepta-*O*-acetyl-α-cellobiosyl bromide (760 mg, 0.95 mmol) and silver oxide (760 mg) were added to a mixture of 2,5-dinitrophenol (769 mg, 4.13 mmol) in $CH_3CN$ (10 mL) and stirred over drierite at room temperature for ~24 h. The reaction mixture was filtered and the solvent evaporated *in vacuo*. Acetylated 2″,5″-dinitrophenyl cellobioside was purified by flash column chromagraphy using ethyl acetate/hexanes (3:2), crystallized from ethyl acetate/ethanol, and fully characterized by $^1$H NMR and elemental analysis. Deacetylation of the cellobioside using acetyl chloride in methanol and recrystallization from ethanol yield the product as a white solid (77 mg, 0.15 mmol, 25%): mp 169–171 °C $^1$H-NMR ($CD_3OD$): δ 8.25 (d, *J* 2 Hz, H(6″)), 8.10 (m, H(3″,4″)), 5.40 (d, $J_{1,2}$ 8 Hz, H(1)), 3.25–5.00 (m, H(2–6) and H(1′–6′)). Anal. Calcd for $C_{18}H_{24}O_{15}N2{\cdot}0.5H_2O$: C, 41.78; H, 4.84; N, 5.42. Found: C, 41.94; H, 5.14; N, 5.17.

*Aryl β-[1-$^2$H]cellobiosides* were prepared as follows. 2,3,6,2′,3′,4′,6′-Hepta-*O*-acetyl-D-cellobiose (5 g, 7.85 mmol) dissolved in DMSO (16 mL) and acetic anhydride (10 mL) was stirred at room temperature overnight. The mixture was poured over water and centrifuged and the solvent decanted, leaving a colorless syrup. The syrup was washed in this way 10 times and then dried overnight. The syrup was dissolved in THF (10 mL), sodium borodeuteride (190 mg) in $D_2O$ (1 mL) was added, and the reaction was allowed to proceed at room temperature for 2 h. The solvent was evaporated *in vacuo*, leaving a colorless syrup. 2,3,6,2′,3′,4′,6′-Hepta-*O*-acetyl-[1-$^2$H]cellobiose was purified from the contaminating protio-cellobiose octaacetate which arose from incomplete oxidation, by flash column chromatography using ethyl acetate/hexanes (2:1). The deuterio-hemiacetal was dissolved in THF (5 mL), sodium borodeuteride (30 mg) in $D_2O$ (0.2 mL) was added, and the reaction mixture was stirred overnight at room temperature. After concentration the remaining syrup was dissolved in $CHCl_3$, washed with water (2 × 50 mL), dried over $MgSO_4$ and solvent evaporated *in vacuo*. 1,2,3,6,2′,3′,4′,6′-Octa-*O*-acetyl β-[1-$^2$H]cellobiose was crystallized from ethanol and characterized by $^1$H-NMR. Deuterated aryl β-cellobiosides were synthesized from the deuterated cellobiose octaacetate by exactly the same route as the protio compounds. In all cases, the $^1$H-NMR spectra were identical except for the absence of the anomeric proton, and elemental analyses within the usual error limits were obtained.

*Kinetics.* All steady-state kinetic studies were performed by recording changes in UV/vis absorbance using a Pye-Unicam PU-8800 spectrophotometer equipped with a circulating water bath. Reactions were monitored at wavelengths where there was a convenient absorbance difference between the initial glycoside and the phenol product as previously reported (Kempton & Withers, 1992), using the same extinction coefficients. Initial rates of exoglycanase-catalyzed hydrolysis of aryl β-D-glycosides were determined by incubating solutions of the appropriate substrate concentrations in 50 mM sodium phosphate buffer, pH 7.0, and 0.1% BSA at 37 °C in 1-cm cuvettes within the spectrophotometer until thermally equilibrated. Reactions were initiated by the addition of enzyme, and release of the phenol product was monitored at the appropriate wavelength. Rates were measured for 6–10 different substrate concentrations, which generally ranged from 0.2 to 5 times the $K_m$ except when limited by solubility. Values for $K_m$ and $k_{cat}$ were determined by nonlinear regression analysis using the program *GraFit* (Leatherbarrow, 1990). Enzymatic reaction products were analyzed by thin-layer chromatography (silica gel; ethyl acetate/methanol/water, 7:2:1).

Pre-steady-state kinetic measurements were performed on an Applied Photophysics MV 17 microvolume stopped-flow spectrophotometer equipped with a Grant constant-temperature bath. Reactions were monitored at the same wavelengths as in the steady-state kinetic studies. The concentration of Cex used in each case was chosen to yield a burst with a total absorbance change of 0.06 A. Rates were determined by equilibrating solutions of enzyme and of the appropriate concentrations of substrate in 50 mM sodium phosphate buffer, pH 7.0, to 37 °C. The reactions were monitored by following the release of the phenol product at the appropriate wavelength. Reaction rates were measured at five different substrate concentrations ranging from 0.2 to ~$K_d$ whenever possible. The measurement at each substrate concentration was repeated 3–4 times, and the traces were averaged and fitted to an equation describing a first-order reaction followed by a steady state. This yielded values of the pseudo-first-order rate constant ($k_{obs}$) and the steady state rate at each substrate concentration. Values of $K_d$ and $k_2$ were determined from these $k_{obs}$ values by direct fit to the equation:

$$k_{obs} = \frac{k_2[S]}{K_d + [S]}$$

using the program *Grafit* (Leatherbarrow, 1990).

Isotope effects were determined by comparison of the initial rates of hydrolysis of high (4–7 times the $K_m$ value) concentrations of protio and deuterio substrates determined spectrophotometrically. In most cases, quartz cells were filled with the appropriate concentration of diluted enzyme and incubated at 37 °C, reaction being initiated by the addition of a small volume (50–100 μL) of (thermally equilibrated) substrate. When substrate solubilities precluded this approach, it was necessary to add the enzyme to the preequilibrated substrate. Rates of protio and deuterio substrate hydrolysis were determined in alternation until a total of 8 or 9 rates for each (protio and deuterio) substrate had been measured. Average rates for the protio and deuterio substrates were then calculated, and the ratio was taken to give the isotope effect.

Rates of exoglycanase-catalyzed hydrolysis at different pH values were determined by incubating 2,4-DNPC at different concentrations in the appropriate buffer containing BSA at 37 °C, until thermally equilibrated. The addition of enzyme initiated the reaction, and values for $k_{cat}$ and $K_m$ were determined as previously described. The buffers used were 50 mM citrate (pH 4–6), 50 mM phosphate (pH 6–8), 50 mM Tris (pH 8–9), and 50 mM carbonate–bicarbonate (pH 9–10). In order to determine if the pH had changed during the reaction, the pH of the reaction mixture was measured after recording the rate of hydrolysis. Molar extinction coefficients for 2,4-dinitrophenolate were determined at pH 4.53, 4.78, 5.16, 5.60, and 6.8 as the following: 9.0, 9.6, 10.2, 10.6, and 10.9 $mM^{-1}$ $cm^{-1}$, respectively.

The equilibrium binding constant ($K_i$) and the inactivation rate constant ($k_i$) for 2F-DNPC were measured by incubating the exoglycanase (0.049 mg/mL) in 50 mM sodium phosphate buffer, pH 7.0, containing bovine serum albumin (1 mg/mL) at 37 °C in the presence of inactivator. Concentrations of 2F-DNPC used were 0, 0.036, 0.072, 0.12, 0.22, and 0.36 mM. Aliquots (10 μL) of the inactivation mixture were removed at different time intervals and diluted into reaction cells containing a large volume of substrate (2,4-DNPC) at saturating concentrations, and the residual enzymatic activity was determined by monitoring dinitrophenolate release. Pseudo-first-order rate constants ($k_{obs}$) for inactivation were calculated by direct fit of each curve to a first-order function, and then values of $k_i$ and $K_i$ were determined from these $k_{obs}$ values by nonlinear regression analysis using *GraFit* (Leatherbarrow, 1990).

Samples of inactivated exoglycanase were extensively dialyzed at 4 °C against several changes of 50 mM sodium phosphate buffer, pH 7.0, in order to remove the excess inactivator. Aliquots of the dialyzed inactivated enzyme were added to buffer solutions containing BSA (1 mg/mL) and either buffer alone or 55 mM cellobiose. These solutions were incubated at 37 °C and monitored for return of activity by periodic removal of samples and assaying as described above. Reactivation rates were calculated by fitting these data to first-order curves by nonlinear regression analysis (Leatherbarrow, 1990).

Ion spray mass spectrometry was performed on a PE SCIEX API III ion spray LC/MS system. Samples of labeled and unlabeled enzyme (~10 μg) were injected into the mass spectrometer, and mass spectra were recorded. The stoichiometry of incorporation of the inactivator was determined from the mass difference between the labeled and the unlabeled enzyme samples.

## RESULTS

*Substrate Reactivity.* Aryl β-cellobiosides, aryl β-xylobiosides, and aryl β-D-glucopyranosides were reacted with *C. fimi* exoglycanase and the kinetic parameters for exoglycanase-catalyzed hydrolysis determined. Values of $k_{cat}$ = 43.3 $s^{-1}$ and 39.8 $s^{-1}$; $K_m$ = 0.06 mM and 0.018 mM; $k_{cat}/K_m$ = 720 $mM^{-1}$ $s^{-1}$ and 2200 $mM^{-1}$ $s^{-1}$ were determined for $ONPX_2$ and $PNPX_2$, respectively. Parameters for the glucosides and cellobiosides, along with the p$K_a$ values of the phenol leaving groups, are listed in Tables 1 and II. Values of $k_{cat}$ and $k_{cat}/K_m$ for the glucopyranosides are plotted as functions of the aglycon p$K_a$ in the form of Broensted plots, and in both cases a strong dependence on p$K_a$ is observed with reaction constants of $\beta_{lg}$ = −1 (Figure 1). Equivalent plots of log $k_{cat}$ and log ($k_{cat}/K_m$) *vs* leaving group p$K_a$ for the cellobiosides are shown in Figure 2. In this case $k_{cat}$ values are essentially invariant with p$K_a$ over the range p$K_a$ 4–8, but show a relatively weak dependence at higher p$K_a$ values ($\beta_{lg}$ = −0.3) as reflected in a downward break in the plot. The log ($k_{cat}/K_m$) *vs* p$K_a$ plot reveals a linear, but still relatively weak ($\beta_{lg}$ = −0.3), dependence across the full range of substrates.

Table 1: Michaelis–Menten Parameters for the Hydrolysis of Aryl β-D-Glucosides by *C. fimi* Exoglycanase

| phenol substituent | p$K_a$[a] | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|---|
| 2,4-dinitro | 3.96 | 18.0 | 1.9 | 9.5 |
| 2,5-dinitro | 5.15 | 11.6 | 1.7 | 6.8 |
| 3,4-dinitro | 5.36 | 4.3 | 6.5 | 0.66 |
| 2-chloro-4-nitro | 5.45 | 14.6 | 27.5 | 0.53 |
| 4-nitro | 7.18 | 0.033 | 8.3 | 0.0041 |
| 2-nitro | 7.22 | 0.68 | 66.4 | 0.010 |
| 3-nitro | 8.39 | 0.0024 | 11.6 | 0.00021 |

[a] Phenol p$K_a$ values were taken from Barlin and Perrin (1966), Kortum et al. (1961), Robinson et al. (1960), and Ba-Saif and Williams (1988).

Table 2: Michaelis–Menten Parameters for the Hydrolysis of Aryl β-Cellobiosides by *C. fimi* Exoglycanase

| phenol substituent | p$K_a$[a] | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|---|
| 2,4-dinitro | 3.96 | 12.9 | 0.11 | 117 |
| 2,5-dinitro | 5.15 | 9.0 | 0.21 | 43.0 |
| 3,4-dinitro | 5.36 | 15.6 | 0.19 | 82.0 |
| 4-nitro | 7.18 | 15.8 | 0.60 | 26.3 |
| 2-nitro | 7.22 | 13.0 | 0.71 | 18.3 |
| 3,5-dichloro | 8.19 | 9.6 | 0.92 | 10.4 |
| 3-nitro | 8.39 | 12.8 | 1.3 | 9.9 |
| 4-cyano | 8.49 | 9.0 | 1.1 | 8.1 |
| 4-bromo | 9.34 | 4.2 | 2.0 | 2.0 |

[a] Phenol p$K_a$ values were taken from Barlin and Perrin (1966), Kortum et al. (1961), Robinson et al. (1960), and Ba-Saif and Williams (1988).

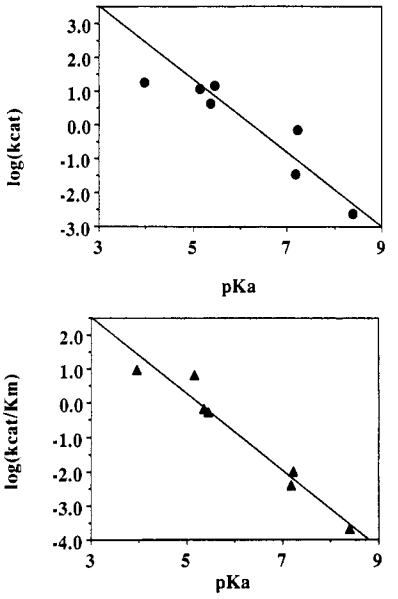



FIGURE 1: Broensted plots relating rates of *C. fimi* exoglycanase-catalyzed hydrolysis of aryl glucosides with the leaving group ability of the phenol. (Upper) Plot of log $k_{cat}$ *vs* p$K_a$ of the aglycon phenol; (lower) plot of the log ($k_{cat}/K_m$) *vs* p$K_a$ of the aglycon phenol.

*Stopped-Flow Studies.* Pre-steady-state kinetics were investigated for five cellobioside substrates, and results are presented in Table 3 and in the form of plots of log $k_2$ and log ($k_2/K_d$) *vs* aglycon p$K_a$ in Figure 3.

*Kinetic Isotope Effects.* Values of the secondary deuterium kinetic isotope effect were measured for two aryl β-D-glucopyranosides and for three aryl β-cellobiosides, and these are shown in Table 4 along with the rate-determining step for each substrate.

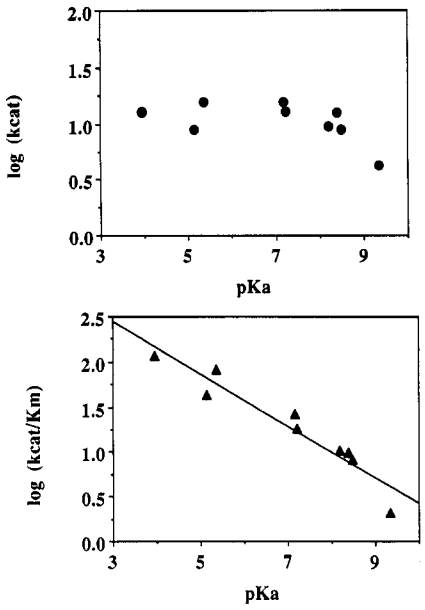



FIGURE 2: 2: Broensted plots relating rates of *C. fimi* exoglycanase-catalyzed hydrolysis of aryl cellobiosides with the leaving group ability of the phenol. (Upper) Plot of log $k_{cat}$ *vs* $pK_a$ of the aglycon phenol; (lower) plot of the log ($k_{cat}/K_m$) *vs* $pK_a$ of the aglycon phenol.

Table 3: Pre-Steady-State Parameters for Hydrolysis of Aryl Cellobiosides by *C. fimi* Exoglycanse

| phenol substituent | $pK_a$ | $k_2$ ($s^{-1}$) | $K_d$ ($mM^{-1}$) | $k_2/K_d$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|---|
| 2,4-dinitro | 3.96 | 1660 ± 236 | 10.0 ± 2.4 | 166 |
| 2,5-dinitro | 5.15 | 1160 ± 94 | 16.2 ± 2.0 | 72 |
| 3,4-dinitro | 5.36 | 626 ± 42 | 5.3 ± 0.7 | 118 |
| 4-nitro | 7.18 | 244 ± 67 | 12.4 ± 4.9 | 20 |
| 2-nitro | 7.22 | ND | ND | 13 |

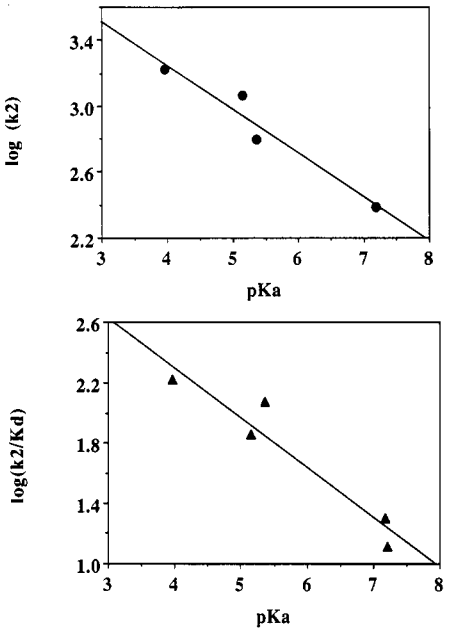



FIGURE 3: Broensted plots relating pre-steady-state rates of *C. fimi* exoglycanase-catalyzed hydrolysis of aryl cellobiosides with the leaving group ability of the phenol. (Upper) Plot of log $k_2$ *vs* $pK_a$ of the aglycon phenol; (lower) plot of the log ($k_2/K_d$) *vs* $pK_a$ of the aglycon phenol.

*Inactivation and Reactivation Studies.* Kinetic parameters determined for the inactivation of Cex by 2F-DNPC are $k_i$ = 6.7 × $10^{-2}$ $min^{-1}$, $K_i$ = 0.11 mM, and $k_i/K_i$ = 6.12 × $10^{-1}$ $min^{-1}$ $mM^{-1}$. These compare with parameters for 2F-DNPG of $k_i$ = 2.5 × $10^{-4}$ $min^{-1}$, $K_i$ = 4.5 mM, and $k_i/K_i$ = 5.56 × $10^{-5}$ $min^{-1}$ $mM^{-1}$ (Tull et al., 1991). Rates of reactivation of 2-deoxy-2-fluorocellobiosyl-Cex in buffer alone and in the presence of 55 mM cellobiose are $k_{react}$ = 8.5 × $10^{-6}$ $min^{-1}$ and $k_{react}$ = 1.9 × $10^{-5}$ $min^{-1}$, respectively. These compare with values of $k_{react}$ = 1.3 × $10^{-5}$ $min^{-1}$ and $k_{react}$ = 4.4 × $10^{-5}$ $min^{-1}$ for 2-deoxy-2-fluoroglucopyranosyl-Cex under equivalent conditions. Figure 4 shows the reconstructed electrospray mass spectrum of Cex before and after inactivation by both 2F-DNPG and 2F-DNPC. Masses of 47 114 ± 7, 47 279 ± 7, and 47 441 ± 7 Da are seen in the three cases corresponding to unlabeled Cex, 2F-glucosyl-Cex, and 2F-cellobiosyl-Cex, respectively. The sample treated with 2F-DNPG was only approximately 50% inactivated, hence the two species observed.

Table 4: Secondary Deuterium Kinetic Isotope Effects Measured with *C. fimi* Exoglycanase

| substrate | RDS[a] | $k_{cat}^H/k_{cat}^D$ |
|---|---|---|
| 2′,4′-dinitrophenyl glucoside | degly | 1.12 ± 0.02 |
| 4′-nitrophenyl glucoside | gly | 1.12 ± 0.02 |
| 2″,4″-dinitrophenyl cellobioside | degly | 1.10 ± 0.02 |
| 4″-nitrophenyl cellobioside | degly | 1.10 ± 0.02 |
| 4″-bromophenyl cellobioside | gly | 1.06 ± 0.02 |

[a] RDS, rate-determining step.

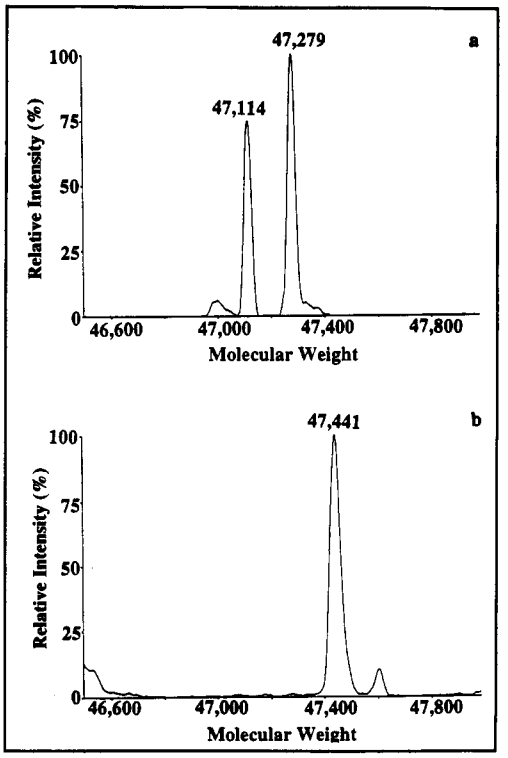



FIGURE 4: Electrospray mass spectrometry of *C. fimi* exoglycanase. (a) Reconstructed mass spectrum of unlabeled and 2F-DNPG-inactivated exoglycanase; (b) reconstructed mass spectrum of 2F-DNPC-inactivated exoglycanase.

*pH Study.* The pH dependence of Cex was investigated using 2,4-DNPC as substrate over a pH range of 4.5–9.4. Values for $k_{cat}$ and $K_m$ were determined and are presented as plots of $k_{cat}$ *vs* pH in Figure 5. Values of $k_{cat}$ are seen to be independent of pH over this range, while $k_{cat}/K_m$ is seen to be dependent upon two ionizations of $pK_a$ = 4.1 and 7.7. The higher $pK_a$ value is a reliable value, but instability of the enzyme at low pH values precluded accurate determination of the lower ionization constant. This value must therefore be taken simply as an estimate.

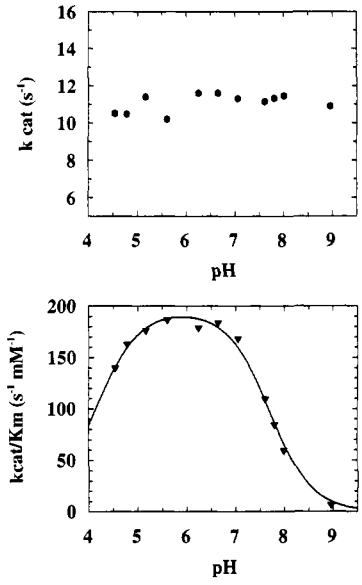



FIGURE 5: pH dependence of the hydrolysis of 2,4-DNPC by *C. fimi* exoglycanase. (Upper) Plot of $k_{cat}$ *vs* pH; (lower) plot of $k_{cat}/K_m$ *vs* pH.

## DISCUSSION

*Proposed Mode of Action.* A double-displacement mechanism as shown in Scheme 1 is assumed for Cex. Indeed, supporting evidence for this has been obtained previously by trapping a covalent 2-deoxy-2-fluoroglucosyl-enzyme intermediate and by subsequent identification of the enzymic nucleophile as Glu233[2] (Tull et al., 1991). This assumption is fully supported and elaborated upon by the data presented in this paper, as follows.

*Substrate Specificity. (a) Xylanase Activity vs Glucanase Activity.* Initial substrate specificity studies showed that Cex was capable of hydrolyzing not only CM-cellulose but also xylan, with comparable efficiency (Gilkes et al., 1984). A reinvestigation using aryl β-xylobiosides ($ONPX_2$ and $PNPX_2$) revealed that $k_{cat}/K_m$ values for xylobioside hydrolysis are in fact 85 times higher than those for equivalent cellobiosides, consistent with the findings of Gilkes et al. 1984. This xylanase activity is not entirely surprising as this exoglycanase has been classified, on the basis of sequence similarities of catalytic domains, as a member of the F family of β-glycanases which is made up largely of xylanases (Beguin, 1990; Gilkes et al., 1991). In addition, both Cex and xylanase Z from *C. thermocellum* [whose cellulase activity is <0.5% of the xylanase activity (Grepinet et al., 1988)] have been shown to be "retaining" glycosidases, thus implying that they share a common catalytic mechanism (Gebler et al., 1992b).

Since cellobiosides differ from xylobiosides by the presence of the C-5 hydroxymethyl groups, it is clear that this group cannot play an essential role in catalysis in the way shown for other glycosidases (Sinnott, 1987; Kempton & Withers, 1992). Indeed, it will be interesting to see how this dual specificity is achieved when the three-dimensional structure is solved (Bedarkar *et al.*, 1992). It seems probable that any hydrogen-bonding interactions present at O-6 when cellobiosides are bound will be satisfied by bound water when xylobiosides are present, as seen for the L-arabinose/D-galactose binding protein (Quiocho, 1989). Other cellulases that have xylanase activity include endoglucanases E and H from *C. thermocellum;* however, in those cases the cellulase activity is considerably greater than that of the xylanase (Hall et al., 1988; Yague et al., 1990).

*(b) Glucosidase Activity vs Glucanase Activity.* Quite significant glucosidase activity is shown by Cex, as has been noted previously (Tull et al., 1991). Such glucosidase activity in other glucanases is very uncommon, but may only be evident with the more reactive glucosides. Comparison of the kinetic parameters for glucosides and cellobiosides shows that Cex hydrolyzes cellobiosides considerably more efficiently than glucosides, consistent with the normal mode of cello-oligosaccharide cleavage in which cellobiose is released.

*pH Dependence.* The substrate employed for these studies was 2,4-DNPC, for which the rate-determining step is hydrolysis of the cellobiosyl-enzyme intermediate (*vide infra*). Interestingly, no dependence upon pH is seen for $k_{cat}$, whereas $k_{cat}/K_m$ depends strongly upon an ionization in its basic limb of $pK_a = 7.7$. This suggests the presence of a group in the free enzyme of $pK_a = 7.7$ which must be in its protonated state to be catalytically active. Binding of substrate, however, shifts the $pK_a$ of this group considerably since no such pH dependence is seen for the enzyme/substrate complex, as indicated by the pH-independent $k_{cat}$ plot. Thus only one protonation state of the enzyme can bind substrate. The identity or specific role of such a group is unclear but could involve a residue responsible for hydrogen bonding to the carboxylate group of Glu233 as it departs from the anomeric center. The ionization of approximate $pK_a = 4.1$ in the acidic limb likely reflects that of the general base catalyst required for this step. The $pK_a$ estimated is indeed similar to that of $pK_a < 5$ found for the same step in *Agrobacterium* β-glucosidase (Kempton & Withers, 1992).

*Substrate Reactivity. (a) β-Glucosidase Activity of C. fimi Exoglycanase.* The strong correlations of both log $k_{cat}$ and log $(k_{cat}/K_m)$ with leaving group $pK_a$ for the glucoside substrates indicate that both the rate-determining step and the first irreversible step in catalysis are the formation of the glycosyl-enzyme, since this is the step in which the C–O bond to the phenolate is cleaved. Stopped-flow studies provided further evidence for this since no pre-steady-state burst was observed for PNPG under conditions where a full burst was observed for PNPC (*vide infra*). Interestingly, however, a burst comparable in magnitude (70% of the maximum value) to that measured for 2,4-DNPC was observed for 2,4-DNPG, indicating that for this substrate deglycosylation is mostly rate-limiting. Indeed, the $k_{cat}$ value for 2,4-DNPG falls slightly below the line defined by the other substrates in the Broensted plot as might be expected were this to be the case. The large value of the reaction constant, $\beta_{lg} = -1$ (calculated without the value for 2,4-DNPG included), which is similar to those found for several β-glucosidases using aryl glucopyranoside substrates [for example, $\beta_{lg} = -0.7$ for both *Agrobacterium sp.* β-glucosidase (Kempton & Withers, 1992) and sweet almond β-glucosidase (Sinnott, 1990)] reflects a large degree of negative charge accumulation on the phenolate oxygen at the glucosylation transition state. This indicates that there is almost complete C–O bond cleavage and relatively little proton donation.

The secondary deuterium kinetic isotope effect measured for PNPG ($k_H/k_D = 1.12$) is consistent with this view since this value reflects substantial $sp^3$ to $sp^2$ rehybridization, thus a large degree of oxocarbonium ion character at the glucosylation transition state (Knier & Jencks, 1980) and necessarily, therefore, considerable bond cleavage. This isotope

[2] The active site nucleophile of Cex was originally numbered as Glu274 on the basis of the sequence of the intact enzyme containing the leader sequence (Tull et al., 1991). In this paper, and in all future work, the numbering scheme will be that of the mature, processed enzyme.

effect is larger than those reported for the glycosylation transition states in many other β-glycosidases, values tending to range from 1.0 for *E. coli (lac z)* β-galactosidase to 1.10 for other glycosidases (Dahlquist et al., 1969; Legler et al., 1980; van Doorslaer at al., 1984; Kempton & Withers, 1992). However, interpretations are clouded somewhat by the known dependence of the kinetic isotope value on the nature of the leaving group involved in each case. In combination with the large $\beta_{lg}$ values this would indicate a glycosylation transition state for these substrates with considerable charge development, little or no protonic assistance, and relatively little preassociation of the enzymic carboxylate nucleophile. Interestingly, the isotope effect for 2,4-DNPG ($k_H/k_D$ = 1.12) is also large, but falls well within the range measured for other glycosidases on the deglycosylation step [$k_H/k_D$ = 1.09 for *Botrydiplodia theobromae* β-glucosidase (Umezerike, 1988) to $k_H/k_D$ = 1.2–1.25 for *E. coli (lac z)* β-galactosidase (Sinnott, 1978)].

*(b) Glucanase Activity of C. fimi Exoglycanase.* Broensted correlations with the cellobiosides are quite different. The absence of any significant dependence of log $k_{cat}$ upon the leaving group p$K_a$ across most of the range indicates that glycosylation is not rate-limiting with these substrates. Rather, hydrolysis of the cellobiosyl-enzyme is the most probable rate-determining step for these more active cellobiosides. A downward break at higher p$K_a$ values suggests that the rate-determining step changes from deglycosylation to glycosylation with the poorer leaving groups, though the relatively weak dependence ($\beta_{lg}$ = –0.3) of this portion obscures this behavior. Such biphasic Broensted plots are not uncommon with glycosidases since similar, though more pronounced, behavior has been seen when probing substrate reactivity of glucosidases with aryl β-D-glucopyranosides (Dale et al., 1986; Sinnott, 1990; Kempton & Withers, 1992).

By contrast, the Broensted plot for log ($k_{cat}/K_m$) shows a modest ($\beta_{lg}$ = –0.3) linear dependence across the entire p$K_a$ range. Since $k_{cat}/K_m$ reflects the first irreversible step, likely the initial bond cleavage, this dependence on leaving group ability is expected. The stopped-flow data in Figure 3 provide further insight into this glycosylation step. Good correlations are observed in both the $k_2$ and $k_2/K_d$ plots, with slopes of $\beta_{lg}$ = –0.3. This is again completely consistent with the slope of the p$K_a$-dependent region of the $k_{cat}$ plot for these substrates ($\beta_{lg}$ = –0.3), as expected if both reflect the formation of the cellobiosyl-enzyme. This slope is considerably less than that seen for the glucosides ($\beta_{lg}$ = –1), reflecting some degree of charge buildup on the phenolate oxygen, but not as much as seen for the glucosides. There are two likely causes for this difference. One could be that there is less C–O bond cleavage at the cellobiosylation transition state than at that for glucosylation. The other could be that general acid catalysis is more efficient in cellobioside hydrolysis, resulting in more proton donation than was seen for the glucosides. It is not easy to distinguish between these two possibilities.

The identical secondary deuterium kinetic isotope effects measured for the more reactive cellobiosides (2,4-DNPC and PNPC), $k_H/k_D$ = 1.10, indicate substantial oxocarbonium ion character at the deglycosylation transition state, suggesting that there is relatively little preassociation of the water. This value is comparable to those reported for this step with other "retaining" β-glycosidases [$k_H/k_D$ = 1.11 for *Agrobacterium sp.* β-glucosidase (Kempton & Withers, 1992) and $k_H/k_D$ = 1.2–1.25 for *E. coli (lac z)* β-galactosidase (Sinnott, 1978)], thus providing further evidence for the involvement of a covalent glycosyl-enzyme intermediate during glycoside hydrolysis since such secondary deuterium kinetic isotope effects could only be seen if the intermediate had more $sp^3$ character than the subsequent transition state. By contrast, the smaller isotope effect ($k_H/k_D$ = 1.06) measured for the cellobiosylation step using 4-BrPC indicates a transition state for that substrate with lesser oxocarbonium ion character.

*Inactivation and Reactivation Studies.* 2-Deoxy-2-fluoro-β-D-glycosides have been used to inactivate several glycosidases through trapping of covalent glycosyl-enzyme intermediates (Withers et al., 1990; Gebler et al., 1992a; Wang et al., 1993). The presence of the electronegative fluorine at C-2 destabilizes the oxocarbonium ion-like transition states, thus slowing down both the glycosylation and deglycosylation steps while a good leaving group such as 2,4-dinitrophenolate accelerates glycosylation, resulting in accumulation of the intermediate. The inactivation of the exoglycanase was probed using two such inactivators, 2F-DNPC and 2F-DNPG. As seen in Figure 4, electrospray mass spectrometric analysis revealed that in both cases 1 mol of inactivator was incorporated per mole of enzyme. Formation of the 2-deoxy-2-fluorocellobiosyl-enzyme is $10^4$-fold more efficient than formation of the 2-deoxy-2-fluoroglucosyl-enzyme, as indicated by relative $k_i/K_i$ values. Interestingly, this rate ratio is greater than that seen in relative $k_{cat}/K_m$ values for the equivalent parent substrates. This likely reflects the fact that highly charged transition states will be more sensitive to fluorine substitution than ones with less charge. Thus a greater discrimination might be expected with fluoro sugars than with their parent substrates. As noted earlier, both secondary deuterium kinetic isotope effects and $\beta_{lg}$ values point to a more highly charged transition state for glucosylation than for cellobiosylation, completely consistent with these observations.

Turnover of the 2-fluoroglucosyl- and 2-fluorocellobiosyl-enzymes, and therefore, reactivation *via* release of free enzyme, can occur by two separate routes, by hydrolysis or by transglycosylation to a suitable acceptor such as cellobiose. These two routes have been demonstrated previously for *Agrobacterium* β-glucosidase (Withers & Street, 1988) and for *E. coli* β-galactosidase (McCarter et al., 1992). Reactivation rates for the two 2-fluoroglycosyl-enzymes are very similar, indicating that, in strict contrast to the situation with the glycosylation step, the presence of the distal glucose moiety does *not* assist the deglycosylation process. Exactly the same situation is found for the substrates since $k_{cat}$ values for 2,4-DNPC and 2,4-DNPG, both substrates for which deglycosylation is rate-limiting, are very similar. Thus in this case, the presence of the fluorine substituent leads to no additional rate discrimination for cellobiosides *vs* glucosides. This would suggest that the two deglycosylation transition states have very similar degrees of positive charge development. Indeed, this is reflected in the very similar secondary deuterium kinetic isotope effects measured for 2,4-DNPC and 2,4-DNPG.

*Comparison of Transition-State Structures.* The transition states for hydrolysis of the glucosyl-enzyme and the cellobiosyl-enzyme are very similar and involve substantial bond cleavage and positive charge development at the anomeric center. In contrast, the transition states for formation of the intermediate seem to be quite different in the two cases. The transition state for formation of the glucosyl-enzyme from PNPG is relatively late, with almost complete bond cleavage, little protonic assistance, and little preassociation of the enzymic nucleophile. In contrast, the cellobiosylation transition state is relatively early with either little bond cleavage or consider-

able protonic assistance coupled with substantial nucleophilic preassociation.

*Conclusions.* All the data reported here are consistent with a double-displacement mechanism for this cellulase in which a glycosyl-enzyme intermediate is formed and hydrolyzed with acid/base catalytic assistance *via* oxocarbonium ion-like transition states. Specificity for hydrolysis *via* cleavage of cellobiosyl rather than glucosyl units is provided by interactions with the distal sugar moiety which accelerate the glycosylation step but have no significant effect on the deglycosylation step. The combination of smaller $\alpha$-deuterium kinetic isotope effects and lesser charge development on the phenolate oxygen for cellobiosyl-enzyme formation compared to that for glucosyl-enzyme formation would suggest that this results in an earlier transition state for formation of the cellobiosyl-enzyme. This likely arises from increased protonic assistance, possibly coupled with a greater degree of nucleophilic preassociation by the enzymic nucleophile. The energy input required for formation of this more highly organized transition state is presumably derived from additional binding interactions with the second sugar moiety.

## ACKNOWLEDGMENT

We thank Drs. Gilkes, Kilburn, and Warren for supplying the exoglycanase, Drs. Aebersold and Miao for assistance with electrospray mass spectrometry, Mr. Trevor Andrews, Ms. Carola Doray, and Ms. Karen Rupitz for assistance with steady-state kinetic experiments, and Dr. Qingping Wang for assistance with stopped-flow studies.

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