Mechanistic Consequences of Mutation of Active Site Carboxylates in a Retaining β-1,4-Glycanase from *Cellulomonas fimi*†

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ABSTRACT: The exoglucanase/xylanase Cex from *Cellulomonas fimi* is a retaining glycosidase which functions *via* a two-step mechanism involving the formation and hydrolysis of a covalent glycosyl-enzyme intermediate. The roles of three conserved active site carboxylic acids in this enzyme have been probed by detailed kinetic analysis of mutants modified at these three positions. Elimination of the catalytic nucleophile (E233A) results in an essentially inactive enzyme, consistent with the important role of this residue. However addition of small anions such as azide or formate restores activity, but as an inverting enzyme since the product formed under these conditions is the α-glycosyl azide. Shortening of the catalytic nucleophile (E233D) reduces the rates of both formation and hydrolysis of the glycosyl-enzyme intermediate some 3000−4000-fold. Elimination of the acid/base catalyst (E127A) yields a mutant for which the deglycosylation step is slowed some 200−300-fold as a consequence of removal of general base catalysis, but with little effect on the transition state structure at the anomeric center. Effects on the glycosylation step due to removal of the acid catalyst depend on the aglycon leaving group ability, with minimal effects on substrates requiring no general acid catalysis but large ($>10^5$-fold) effects on substrates with poor leaving groups. The Brønsted β_{1g} value for hydrolysis of aryl cellobiosides was much larger ($\beta_{1g} \sim -1$) for the mutant than for the wild-type enzyme ($\beta_{1g} = -0.3$), consistent with removal of protonic assistance. The pH-dependence was also significantly perturbed. Mutation of a third conserved active site carboxylic acid (E123A) resulted in rate reductions of up to 1500-fold on poorer substrates, which could be largely restored by addition of azide, but without the formation of glycosyl azide products. These results suggest a simple strategy for the identification of the key active site nucleophile and acid/base catalyst residues in glycosidases without resort to active site labeling.

Cellulases and xylanases, part of a larger group of glycosyl-hydrolases, hydrolyze β-1,4-glycosidic linkages in cellulose and xylan. The amino acid sequences, deduced from the sequences of the genes encoding them, are known for about 200 enzymes from a variety of bacterial, fungal, and plant sources (Henrissat & Bairoch, 1993). The potential for use of the enzymes in biomass degradation and fuel production has spurred studies of individual substrate-binding and catalytic domains, of synergism between enzymes, and of catalytic mechanisms (Sinnott 1990; Beguin & Aubert, 1994).

Cellulases and xylanases are grouped into several families on the basis of amino acid sequence similarities in their catalytic domains (Henrissat & Bairoch, 1993). Some of these families contain both endo- and exo-β-1,4-glycanases, suggesting that their catalytic domains have somewhat different topologies. However, all members of a particular family appear to hydrolyze their substrates by one of two distinct catalytic mechanisms (Gebler *et al*., 1992): the configuration at the anomeric carbon is either retained following hydrolysis or inverted. In retaining enzymes, the mechanism involves a double displacement in which a covalent α-D-glycopyranosyl-enzyme intermediate is formed and hydrolyzed via oxocarbenium ion-like transition states as shown in Scheme 1 (Sinnott, 1990; Tull & Withers, 1994).

The β-1,4-glycanase Cex[1] is one of a number of cellulases and xylanases from *Cellulomonas fimi*, the genes for which have been cloned, sequenced, and expressed in *E. coli* (Meinke *et al*., 1994; Shen *et al*., 1994). Cex, 47.1 kDa, comprises an N-terminal catalytic domain and a C-terminal cellulose binding domain connected by a proline-threonine-rich linker region (O'Neill *et al*., 1986a). It is a member of family 10 (formerly family F) of glycosyl-hydrolases (Gilkes *et al*., 1991; MacLeod *et al*., 1994). Like other members of family 10, Cex hydrolyzes both xylan and, to a lesser extent, (carboxymethyl)cellulose (CMC) (Gilkes *et al*., 1984; Tull & Withers, 1994). The activity on CMC appears to be predominantly exohydrolytic (Gilkes *et al*., 1984; Shen *et al*., 1995). Cex also hydrolyzes a range of soluble aryl xylobiosides, cellobiosides, and glucosides (Gilkes *et al*.,

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[1] Abbreviations: Abg, *Agrobacterium faecalis* β-glucosidase; Cex, *Cellulomonas fimi* exoglycanase; ESIMS, electrospray ionization mass spectrometry; 2,4-DNPC, 2,4-dinitrophenyl β-cellobioside; 3,4-DNPC, 3,4-dinitrophenyl β-cellobioside; 4-CNPC, 4-cyanophenyl β-cellobioside; 4-BrPC, 4-bromophenyl β-cellobioside; 2,4-DNPG, 2,4-dinitrophenyl β-glucoside; 3,4-DNPG, 3,4-dinitrophenyl β-glucoside; 2,5-DNPG, 2,5-dinitrophenyl β-glucoside; 2-Cl-4-NPG, 2-chloro-4-nitrophenyl β-glucoside; PNPC, 4-nitrophenyl β-cellobioside; ONPC, 2-nitrophenyl β-cellobioside.

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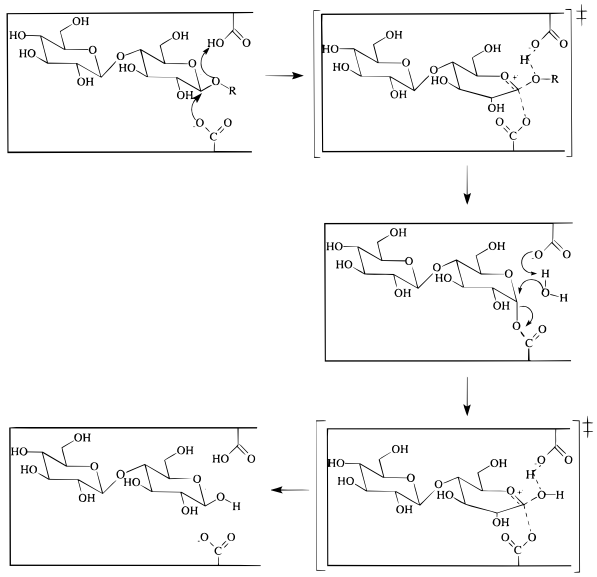
Scheme 1



1991; Tull & Withers, 1994). Cex is a retaining enzyme (Gebler *et al*., 1992), the catalytic nucleophile being Glu 233 as identified by labeling with a mechanism-based inactivator (Tull *et al*., 1991). The acid/base catalyst is Glu 127, identified by a detailed kinetic analysis of mutants generated by site-directed mutation (MacLeod *et al*., 1994). The crystal structure of the catalytic domain of Cex verifies the assignment of Glu 233 and Glu 127 as the nucleophile and acid/base catalyst, respectively (White *et al*., 1994, 1996). These glutamates, conserved in all members of family 10 (MacLeod *et al*., 1994), are positioned within the active site cavity with the carboxyl groups 5.5 Å apart. The distance between the carboxylates is that expected for retaining β-1,4-glycanases (Wang *et al*., 1994; White *et al*., 1994, 1996; Harris *et al*., 1994; Derewenda *et al*., 1994; Wakarchuk *et al*., 1994; McCarter & Withers, 1994).

Cex is one of the few β-1,4-glycanases for which details of the mechanism are known (Tull & Withers, 1994). In addition, it is the only glycosidase for which a crystal structure of the catalytically competent intermediate formed on the wild-type enzyme has been determined (White *et al*., 1996). This crystal structure provides the insights needed for a detailed examination of the roles of the active site carboxylates, as described in this paper.

MATERIALS AND METHODS

Reagents. Growth media components were from Difco. Restriction endonucleases, DNA ligase, and polymerases were from Pharmacia and BRL. Radionucleotides were from New England Nuclear Corp. Buffer chemicals were obtained from Sigma and BDH. 2,4-DNPC, 3,4-DNPC, 4-CNPC, 4-BrPC, 2,4-DNPG, 2,5-DNPG and 3,4 DNPG were synthesized as described elsewhere (Tull & Withers, 1994). 2-Cl-4NPG was a generous gift from Dr. Marc Claeyssens. PNPC and ONPC were obtained from Sigma Chemical Co.

Production and Purification of Cex Mutants. Site-directed mutations of active site carboxylates of Cex were generated by the method of Kunkel (1987) using 25mer oligonucleotide primers (synthesized by the University of British Columbia Nucleic Acid and Peptide Synthesis Unit). The primers used to generate the mutations were as follows, with the positions of the mismatches underlined: E233D: 5′P-G CGC ATC ACC GAC CTC GAC ATC CGC-3′; E233A: 5′P-G CGC ATC ACC GCG CTC GAC ATC CGC-3′; E127A: 5′P-AC GTC GTC AAC GCG GCG TTC GCC GA-3′; D123A: 5′P-TC GCG TCG TGG GCC GTC GTC AAC GAG-3′. Mutations were confirmed by DNA sequencing. DNA fragments containing the mutations were subcloned to pUC12-1.1Cex(PTIS) (O'Neill *et al*., 1986b), replacing the corresponding wild-type fragment. The plasmid DNA was transformed to *E. coli* JM101 (Hanahan *et al*., 1983) for expression of the mutant *cex* gene.

Wild-type Cex and mutants of Cex were produced in *E. coli* and purified from whole-cell extracts by affinity chromatography on CF-1 cellulose (Sigma) as described previously (MacLeod, *et al*., 1994). Contamination of E233A by wild-type enzyme was demonstrated by incubating E233A (18.9 mg·mL^{-1}) with 2,4-dinitrophenyl-2-deoxy-2-fluoro β-cellobioside (2.9 mM) in pH 7.0 buffer and removing aliquots (10 μL) at intervals and assaying for residual activity with 2,4-DNPC (1.2 mM) in a 0.15 mL cell. A sample of the inactivated enzyme mixture was then subjected to electrospray mass spectrometric analysis on a PE-Sciex API-300 instrument.

Steady-State Kinetics. Michaelis−Menten parameters for aryl cellobiosides, glucosides, and $PNPX_2$ were determined by continuous measurement at the appropriate wavelength of the release of the substituted phenol product using either a Pye-Unicam PU8700 or a Hitachi U 2000 spectrophotometer, essentially as described previously (Kempton & Withers, 1992; Tull & Withers, 1994). Phenol pK_a values were taken from Barlin and Perrin (1966), Kortum *et al*. (1961), Robinson *et al*. (1960), and Ba-Saif and Williams (1988).

Reaction mixtures, in 50 mM phosphate buffer (pH 7.0), 37 °C unless otherwise indicated, were preincubated in the cell-holder at the appropriate temperature for 10 min prior to addition of enzyme. Sodium azide or sodium formate was included in mixtures as indicated. Enzyme-catalyzed hydrolysis for a particular substrate was measured at 8 to 10 different substrate concentrations ranging from about $0.14K_m$ to $7K_m$, where practical. Values for K_m and k_{cat} were determined from the initial rates of hydrolysis (v_o) *vs* substrate concentration, by nonlinear regression analysis using the computer program GraFit (Leatherbarrow, 1990).

Pre-Steady-State Kinetics. Measurements were made with an Applied Photophysics MV 17 microvolume stopped-flow spectrophotometer equipped with a Grant constant temperature bath. The concentration of enzyme used in each case was chosen to produce an increase in absorbance of 0.06. The buffer was 50 mM phosphate, pH 7.0. Reaction rates were measured 3−4 times at five different substrate concentrations ranging from $0.2K_d$ to $\sim K_d$ whenever possible. The traces were averaged, and then fitted to an equation describing a first-order reaction followed by a steady state, giving 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 k_{obs} values by direct fit to the equation:

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

using the program GraFit (Leatherbarrow, 1990). Standard errors were calculated using the same program.

Secondary Deuterium Kinetic Isotope Effects. Isotope effects were determined spectrophotometrically by comparison of the initial rates of hydrolysis of high ($4-7 \times K_m$) concentrations of protio and deuterio substrates. In most cases, quartz cells were filled with the appropriate concentration of diluted enzyme and equilibrated at 37 °C; the reaction was initiated by the addition of a small volume (50−100 μL) of thermally equilibrated substrate. When substrate solubilities precluded this approach, enzyme was added to the preequilibrated substrate. Rates of protio and deuterio substrate hydrolysis were determined alternately. Average rates for the protio and deuterio substrates were then calculated and the ratios taken to give the isotope effect.

Effect of pH on Enzyme Kinetics. With the wild-type enzyme or the E233D mutant, different concentrations of 2,4-DNPC in the appropriate buffer containing 1 mg/mL BSA and 145 mM NaCl were equilibrated at 37 °C. Reactions were initiated by addition of enzyme. Dinitrophenolate release was monitored at 400 nm using the appropriate extinction coefficients (Kempton & Withers, 1992). Values for k_{cat} and K_m were determined as described above. The dependence of k_{cat} on pH for E127A was determined as follows. Saturating concentrations of 2,4-DNPC ($75K_m$) in the appropriate buffer containing 1 mg/mL BSA and 145 mM NaCl were equilibrated at 37 °C. Reactions were initiated by addition of enzyme. Values of k_{cat} were then calculated from the initial rates of hydrolysis. Values of K_m at the pH extremes were determined to ensure that saturating conditions had been maintained. The pH dependence of k_{cat}/K_m for the E127A mutant was determined by incubating PNPC at a final concentration of $0.2K_m$ in the appropriate buffer as above. The reactions were initiated by the addition of enzyme. Release of 4-nitrophenolate was measured at 400 nm until the substrate was depleted. The change in absorbance with time was fitted to a first-order rate equation using the program GraFit (Leatherbarrow, 1990), yielding values for the pseudo-first-order rate constant at each pH value. Since at low substrate concentrations ($S \ll K_m$), the reaction rates are given by the equation:

$$v = \frac{k_{cat}[E][S]}{K_m}$$

the k_{obs} values correspond to $[E]k_{cat}/K_m$; thus, k_{cat}/K_m values can be extracted. The pH of each reaction mixture was measured following hydrolysis to ensure that it had not changed.

Analysis of the Products of Enzymatic Hydrolysis. Mixtures containing 3.5 mM 2,4-DNPC, 2 M azide, and 0.8 mg/mL mutant enzyme in 50 mM phosphate buffer (pH 7.0) were incubated at 37 °C overnight. Reaction mixtures and standards (2 μL) were applied to a 0.2 mm silica gel aluminum TLC plate (#60 F254; E. Merck) and allowed to air-dry. The solvent was a mixture of ethyl acetate/methanol/water (7:2:1 v/v/v). After development, the chromatograms were air-dried for 5 min, then dipped in a solution of 10% H_2SO_4 in methanol, and heated until the charred reaction products were visible.

^{1}H NMR Spectrometry of the Products of Enzymatic Hydrolysis. Reaction mixtures containing 4.5 mg of 2,4-DNPC, 2 M azide, and 0.4 mg/mL E233A were incubated at 37 °C overnight. Enzyme was removed by passing the solution through a 10 kDa cutoff polysulfone membrane (ultrafree-MC; Millipore). Samples of the filtrate were prepared for ^{1}H NMR analysis by repeated freeze-drying and dissolving in D_2O. Spectra were recorded with a Bruker 400 MHz spectrometer.

Table 1: Michaelis−Menten Parameters for the Hydrolysis of Aryl β-D-Cellobiosides and Aryl β-Glucosides by Cex E233D

phenol substituent	pK_a	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
cellobiosides				
2,4-dinitro	3.96	0.086 ± 0.006	0.0031 ± 0.0001	0.036
3,4-dinitro	5.36	0.10 ± 0.004	0.0027 ± 0.0001	0.027
4-nitro	7.18	0.66 ± 0.03	0.0038 ± 0.0001	0.0058
2-nitro	7.22	0.62 ± 0.02	0.0030 ± 0.0001	0.0048
4-cyano	8.49	1.6 ± 0.4	0.0028 ± 0.0005	0.0018
4-bromo	9.34	1.2 ± 0.3	0.0025 ± 0.0002	0.0021
glucosides				
2,4-dinitro	3.96	1.35 ± 0.09	0.0035 ± 0.0001	0.0026
2,5-dinitro	5.15	1.96 ± 0.07	0.0039 ± 0.0001	0.0020
3,4-dinitro	5.36	5.50 ± 2.0	0.0010 ± 0.0004	0.00018
2-chloro-4-nitro	5.45	20.2 ± 1.6	0.0018 ± 0.0001	0.00009

RESULTS AND DISCUSSION

Activities of the Mutant Enzymes. Kinetic parameters for the hydrolysis of a number of aryl glycosides by the E233D, E127A, and D123A mutants are presented in Tables 1−4. The fact that only very low activities are observed in some cases raises concerns that the activities actually measured are true activities of the mutants and not due to contamination, from whatever source, possibly translational misincorporation (Schimmel, 1989). Indeed, in the case of the nucleophile mutant E233A, the activity measured (10^4-fold lower than wild-type) was shown to be due to contamination by wild-type enzyme, as described more fully later. This is not the case, however, for the nucleophile mutant E233D since it is not significantly inactivated by the mechanism-based inhibitor 2,4-dinitrophenyl-2-deoxy-2-fluoro β-cellobioside under conditions which would completely inactivate the wild-type enzyme (Tull & Withers, 1994). E233D also has a different pH profile from that of the wild-type enzyme. E127A *is* inactivated by 2F-DNPC, as would be expected, so this criterion cannot be applied. However, there is a very marked difference in substrate reactivity between E127A and the wild-type enzyme which is fully consistent with loss of acid/base catalytic assistance (MacLeod *et al*., 1994).

Reducing the Length of the Catalytic Nucleophile: E233D. Review of the data in Table 1, along with the following parameters for hydrolysis of $PNPX_2$ ($k_{cat} = 0.011 \pm 0.001$ s^{-1}, $K_m = 0.018 \pm 0.001$ mM, $k_{cat}/K_m = 0.61$ s^{-1} mM^{-1}), reveals that, just as with the wild-type enzyme (Tull & Withers, 1994), the best substrates are the xylobiosides followed by the cellobiosides and then the glucosides. Thus, even though the rates of hydrolysis are slowed enormously by the mutation, the order of reactivity is retained. In order to assess the kinetic consequences of moving the catalytic nucleophile approximately 1 Å from its position in the wild-type enzyme, it is first necessary to determine which is the rate-determining step for this mutant. Insights into the identities of the rate-determining steps and the transition state structures for hydrolysis of the aryl cellobiosides and aryl glucosides by E233D have therefore been obtained by studying the dependence of kinetic parameters upon the aglycon leaving group ability.

Values of k_{cat} for hydrolysis of the cellobiosides and glucosides were found to be essentially independent of the

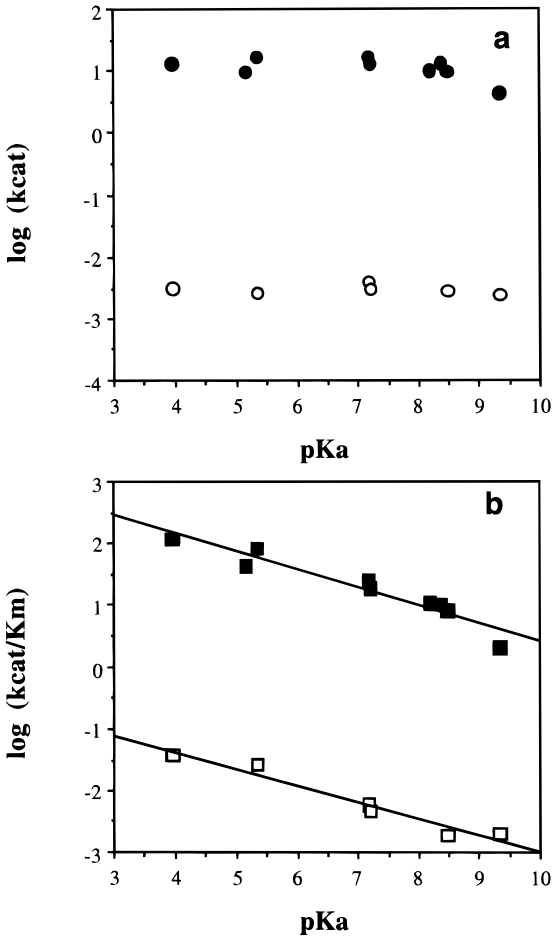


FIGURE 1: Brønsted plots relating rates of hydrolysis of aryl cellobiosides by *C. fimi* exoglycanase (filled points) and its E233D mutant (open points) with the phenol leaving group ability.

pK_a values for the phenolic leaving groups over the range pK_a = 4−9 (Table 1; Figure 1). Thus, it would appear that the deglycosylation step is rate-determining for all substrates with this mutant. In the case of the wild-type enzyme, the deglycosylation step was shown to be rate-determining for all substrates except those with aglycon phenols of high pK_a, for which the glycosylation step became rate-determining (Tull & Withers, 1994).

By contrast (Figure 1b), the log (k_{cat}/K_m) Brønsted plot for the wild-type enzyme revealed a real, but relatively weak dependence ($\beta_{1g} = -0.3$, correlation coefficient = 0.94) of log (k_{cat}/K_m) upon the pK_a values across the full range of substrates studied. This is consistent with expectations that k_{cat}/K_m reflects the first irreversible step, *i.e.*, formation of the glycosyl-enzyme. The Brønsted constant for hydrolysis of the aryl-cellobiosides by E233D ($\beta_{1g} = -0.3$) is identical to that for the wild-type enzyme (Tull & Withers, 1994). Interestingly, the Brønsted constants for the hydrolysis of aryl β-glucosides by Abg, a β-glucosidase from *Agrobacterium faecalis*, and its catalytic nucleophile mutant, E358D, while larger than that seen in this study ($\beta_{1g} = -0.7$; Withers *et al*., 1992), are also identical, as also are β_{1g} values for wild-type and E78D *Bacillus subtilis* xylanase (Lawson *et al*., 1996). Thus, in all three cases, the shortening of the nucleophile carboxylate does not affect the degree of negative charge development on the departing phenolate at the glycosylation transition state, hence the degree of C−O bond cleavage or proton donation. Further insights into transition state structure are derived from secondary deuterium kinetic

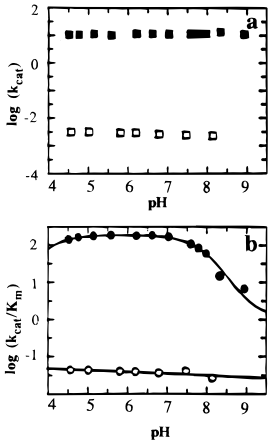


FIGURE 2: Dependence upon pH of kinetic parameters for the hydrolysis of 2,4-DNPC by *C. fimi* exoglucanase (filled points) and its E233D mutant (open points).

isotope effects determined for both wild-type and Cex E233D. The α-secondary deuterium kinetic isotope effect for the hydrolysis of 2,4-DNPC by E233D is $k_H/k_D = 1.09 \pm 0.02$, a value virtually identical to that obtained previously ($k_H/k_D = 1.10 \pm 0.02$) for hydrolysis of this substrate by the wild-type enzyme (Tull & Withers, 1994). Thus, the extent of oxocarbenium ion development at the transition state for hydrolysis of the cellobiosyl-enzyme intermediate is also unaffected by the mutation.

Comparison of k_{cat} values for wild-type and Cex E233D with substrates of aglycon pK_a < 7.5 allow estimation of the effect of this change on the deglycosylation step, k_3, [(4 × 10^3)-fold)], while comparison of k_{cat}/K_m values provides the effect on the glycosylation step, k_2 [(3 × 10^3)-fold)]. These values compare very well to those determined previously for the corresponding nucleophilic Glu to Asp mutant of Abg (Withers *et al*., 1992) and β-galactosidase (LacZ) from *E. coli* (Yuan *et al*., 1994) of (2.5 × 10^3)-fold and [(2−9) × 10^3]-fold, respectively. Assuming that most of this effect is a consequence of changes in the transition state and not in ground state interactions, the rate reduction corresponds to an increase in the glycosylation transition state energy of ∼5 kcal/mol. Estimation of the effect on the deglycosylation transition state is more complex since reducing the length of the catalytic nucleophile would likely strain the covalent cellobiosyl-enzyme intermediate. The value of $\Delta\Delta G^{\ddagger} = 5$ kcal/mol derived from relative k_3 values therefore represents a *minimum* estimate of the actual increase in the deglycosylation transition state energy. Since the increase in energy of the glycosylation transition state is ≈5 kcal/mol, whereas that of the deglycosylation transition state is likely greater than 5 kcal/mol, then mispositioning the nucleophile probably destabilizes the transition state for deglycosylation more than for glycosylation. This interpretation is consistent with deglycosylation being the rate-determining step for E233D with *all* the cellobiosides, whereas for the wild-type enzyme, deglycosylation is rate determining for only the more reactive cellobiosides (e.g., 2,4-DNPC).

Both k_{cat} and k_{cat}/K_m for the hydrolysis of 2,4-DNPC by E233D were independent of pH over the range 4.6−8.4 (Figure 2), as is k_{cat} for the wild-type enzyme, indicating that no active site ionizations which affect hydrolysis rates occur in this pH range. However, in the wild-type enzyme, k_{cat}/K_m is dependent upon one ionization of pK_a = 7.7, and

possibly a second ionization of pK_a around 4. Since this ionization of pK_a = 7.7, reflecting a group which must be protonated to be active, is assigned later (*vide infra*) as that of the acid/base catalyst E127, this suggests that pulling the nucleophile further away raises the pK_a of this group at least 1 unit. This is unlikely to be due to simple electrostatic effects, which would give rise to the opposite response. More likely, it is a consequence of changes in hydrogen bonding interactions in the active site. Inspection of the active site of the wild-type enzyme reveals that Glu 233 is within hydrogen bonding distance of both His 205 and Asn 169; and His 205 is within hydrogen bonding distance of Asp 235 (White *et al*., 1994, 1996). Since Asp 235, His 205, and Glu 233 are highly conserved residues in family 10 β-glycanases, the hydrogen bonding network of Asp235-His205-Glu233 likely plays an important role in maintaining the ionization state of the nucleophile (White *et al*., 1994). Modeling of E233D based on the wild-type structure reveals that neither His 205 nor Asn 169 is now within hydrogen bonding distance of the catalytic nucleophile. The absence of these hydrogen bonding interactions in E233D would modify the environment of the active site, thus altering the pK_as of both the catalytic nucleophile and acid/base catalyst, as shown by the pH profiles.

Elimination of the Nucleophile: E233A, an Inactive Mutant Which Can Be Reactivated. Initial measurements of the apparent k_{cat} value for E233A indicated that replacement of Glu by Ala at the nucleophile position decreased k_{cat} only about (1.3×10^4)-fold for hydrolysis of both PNPC and 2,4-DNPC and K_m values remained relatively unchanged. This finding was inconsistent with equivalent studies on other β-glycosidases where rate decreases of at least 10^6-10^7-fold were observed (Withers *et al.*, 1992; Wang *et al*., 1994; Yuan *et al.*, 1994). Further investigations, however, revealed that this activity was due to wild-type enzyme contaminant. This was demonstrated by measuring the rate of inactivation of the E233A mutant by the mechanism-based inactivator 2,4-dinitrophenyl-2-deoxy 2-fluoro β-cellobioside. Complete inactivation of the enzyme was observed according to a rate constant essentially identical to that measured with the wild-type enzyme under the same conditions (Tull & Withers, 1994). Further, analysis of the enzyme sample treated in this way by electrospray ionization mass spectrometry (ESIMS) revealed no change in mass of the only observable component, the E233A mutant, indicating that the loss of activity could not be due to derivatization of the mutant, and must therefore be due to inactivation of a minor contaminant (<0.01%) of the wild-type enzyme not seen by ESIMS. These results therefore demonstrate that the E233A mutant is essentially inactive, with activity being reduced *at least* 10^6-fold relative to wild-type enzyme.

The inactive E233A mutant can be reactivated, however, by the addition of azide or formate to assay mixtures, as shown in Table 2. No such rate enhancement was observed with E233D or wild-type enzyme. Equivalent behavior has been seen previously with the E358A nucleophile mutant of Abg (Wang *et al.*, 1994), and in that case the activity was shown to arise from the azide binding to the enzyme−substrate complex on the α-face of the substrate in the site formerly occupied by the catalytic nucleophile, then attacking the substrate to form an α-glycosyl azide product. The same result was obtained in this case since, while hydrolysis of PNPC and 2,4-DNPC by wild-type enzyme resulted in the release of cellobiose (Gebler *et al*., 1992), the product released by E233A in the presence of azide was α-cellobiosyl azide, as indicated by TLC, and as confirmed by ^{1}H-NMR spectrometric analysis of the isolated reaction product: ^{1}H NMR (400 MHz, D_2O) δ 5.73 (1 H, d, J = 2.7 Hz), δ 4.0−4.2 (3 H, m), 3.8−4.0 (5 H, m), 3.56−3.79 (4 H, m), δ 3.52 (1 H, t, J = 8.6 Hz). The formation of α-cellobiosyl azide in the presence of azide thus indicates that Cex E233A now acts as an inverting enzyme, as was seen with Abg (Wang et al., 1994). Thus, conversion of a retaining enzyme to an inverting enzyme by elimination of the nucleophile and the inclusion in the reaction of appropriate anions may be generally applicable to glycoside hydrolases. Interestingly, a somewhat complementary experiment involving the substitution of the catalytically innocent Thr 26 by His in the active site of T4 lysozyme altered the catalytic mechanism from inverting to retaining (Kuroki *et al*., 1995).

Table 2: Kinetic Parameters for Hydrolysis of Aryl β-Cellobiosides by Cex E233A and Cex D123A in the Presence and Absence of Anions

enzyme	substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
wild-type[a]	PNPC	11.3 ± 0.1	0.53 ± 0.020	21.3
	2,4-DNPC	7.0 ± 0.07	0.06 ± 0.003	116.4
E233A	PNPC	$<1 \times 10^{-5}$		
	2,4-DNPC	$<1 \times 10^{-5}$		
	2,4-DNPC + 4 M azide	0.024 ± 0.0006	0.26 ± 0.021	0.09
	2,4-DNPC + 4 M formate	0.069 ± 0.0015	0.04 ± 0.003	1.73
D123A	PNPC	0.007 ± 0.0004	18.0 ± 2.3	0.0004
	2,4-DNPC	5.4 ± 0.108	0.53 ± 0.029	10.2
	PNPC + 0.5 M azide	5.0 ± 0.0039	11.0 ± 0.79	0.0076

[a] MacLeod et al. (1994).

Elimination of the Acid/Base Catalyst: E127A. The role of the acid/base catalyst in the first step of the reaction (glycosylation) is donation of a proton (general acid catalysis) to the oxygen of the leaving aglycon to assist the C−O bond cleavage. In the second step of the reaction (deglycosylation), it acts as a general base catalyst, abstracting a proton from water and thereby promoting nucleophilic attack on the glycosyl-enzyme intermediate. Elimination of the acid/base catalyst should therefore affect both steps: however, the extent to which each step is affected should depend on the particular substrate studied. Whereas the deglycosylation rate constant (k_3) for the E127A mutant should be identical for all glycosides of the same sugar, the rate constant for the first step, glycosylation (k_2), should be dependent upon the leaving group ability of the phenol. With activated substrates, *i.e.*, those with a phenol group of low pK_a, acid-catalytic assistance would not be required; thus, k_2 should not be greatly affected, whereas for substrates requiring acid-catalytic assistance of C−O bond cleavage, k_2 should be greatly decreased. Michaelis−Menten parameters for the hydrolysis of a series of aryl cellobiosides are presented in Table 3. As can be seen from these data, and even more clearly in the Brønsted plot of Figure 3, values of k_{cat} for hydrolysis of the cellobiosides by E127A were independent of their phenolic leaving group pK_a values over the range p$K_a \approx 4-7.5$ but dependent on the pK_a at higher values. This indicates that deglycosylation is still rate-limiting for the better substrates (pK_a < 7.5) while glycosylation becomes rate-limiting for the poorer substrates.

Table 3: Michaelis−Menten Parameters for the Hydrolysis of Aryl β-Cellobiosides by Cex E127A

phenol substituent	pK_a	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
2,4-dinitro[a]	3.96	0.0003 ± 0.0001	0.040 ± 0.002	133
3,4-dinitro	5.36	0.0008 ± 0.0002	0.072 ± 0.007	90
4-nitro	7.18	0.020 ± 0.002	0.033 ± 0.001	1.65
2-nitro	7.22	0.021 ± 0.003	0.021 ± 0.001	1.0
3,5-dichloro	8.19	0.15 ± 0.03	0.0027 ± 0.0005	0.018
4-cyano	8.49	0.43 ± 0.01	0.015 ± 0.0001	0.035
4-bromo[a]	9.36	1.9 ± 0.4	(6.6 ± 0.4) × 10^{-4}	3.5 × 10^{-4}

[a] MacLeod et al. (1994).

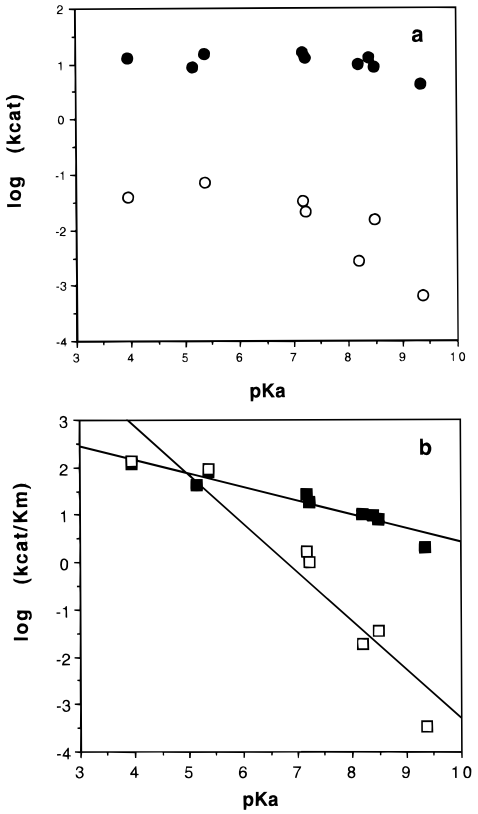


FIGURE 3: Brønsted plots relating rates of hydrolysis of aryl cellobiosides by *C. fimi* exoglucanase (filled points) and its E127A mutant (open points) with the phenol leaving group ability.

Table 4: Pre-Steady-State Parameters for Hydrolysis of Aryl β-Cellobiosides by E127A

phenol substituent	pK_a	k_2 (s^{-1})	K_d (mM)	k_2/K_d (s^{-1} mM^{-1})
2,4-dinitro	3.96	190 ± 7	0.68 ± 0.06	282
3,4-dinitro	5.36	3.3 ± 0.07	0.019 ± 0.002	179
4-nitro	7.18	0.30 ± 0.01	0.17 ± 0.02	1.79

Further insight was obtained by carrying out pre-steady-state kinetic studies on several of these substrates with the E127A mutant, and kinetic parameters so determined are shown in Table 4. All three of these substrates underwent hydrolysis with a significant pre-steady-state burst indicating that deglycosylation was most likely rate-limiting, and allowing the measurement of the kinetic parameters shown. No pre-steady-state burst was seen for the 4-bromophenyl cellobioside. Comparison of the k_2 values obtained both from these stopped-flow measurements and, in the case of the slowest substrates, directly from k_{cat} values, with those of the wild-type enzyme (Tull & Withers, 1994), revealed substantial rate reductions, the extent of which depended upon the leaving group ability of the substrate phenolic moiety. Thus, hydrolysis of substrates such as 2,4-DNPC with good leaving groups is not greatly affected while that of substrates with poorer leaving groups, such as 4-BrPC, is more severely affected. Equivalent effects are also seen in the relative k_{cat}/K_m values which presumably reflect the glycosylation step. Thus, the value of k_{cat}/K_m for hydrolysis of 2,4-DNPC by E127A was essentially unchanged while that for 4-BrPC was reduced ~(3×10^5)-fold relative to wild-type enzyme (MacLeod *et al.*, 1994). This is entirely consistent with Glu 127 being the acid catalyst in Cex. The β-glucosidase from *A. faecalis* behaves in a similar manner when its acid/base catalyst (Glu 170) is eliminated (Wang *et al.*, 1995). This dependence upon leaving group ability is best seen in the Brønsted plot of log (k_{cat}/K_m) versus pK_a shown in Figure 3b. Although there is considerable scatter in the data, or perhaps it is biphasic, it is clear that the reaction catalyzed by the mutant is *much* more dependent on the aglycon leaving group ability (β_{1g} ~−1, $\rho = 0.82$) than is that of the wild-type enzyme ($\beta_{1g} = -0.3$). Such apparent biphasic behavior has been seen previously in plots of log (k_{cat}/K_m) versus pK_a (Kempton & Withers, 1992), and is not easily understood given that a change in the rate-limiting step should not affect this plot. Nonetheless, the greater dependence on leaving group ability implied is also reflected in plots (not shown) of log k_2 and log k_2/K_D *versus* pK_a derived from the pre-steady-state data, where Brønsted constants of $\beta_{1g} \sim -0.8$ and -0.7 were observed. This increase of β_{1g} from -0.3 to -1 indicates a much greater accumulation of negative charge on the oxygen of the departing phenolate in the glycosylation transition state in the mutant. Thus, there is less proton donation to the phenolate oxygen in the mutant, *exactly as would be expected in the absence of the acid catalyst.*

The effects of removal of the general base catalyst on the deglycosylation step are also of interest, and are revealed by comparing wild-type enzyme k_{cat} values for the more reactive cellobiosides (Tull & Withers 1994; MacLeod *et al.*, 1994) with those for the E127A mutant (Table 3). Such a comparison reveals that k_3 is reduced 200−300-fold upon removal of general base catalytic assistance. A much greater (2000-fold) reduction in k_3 values was observed for Abg (Wang *et al.*, 1995), indicating that general base catalysis is more important in that case. Interestingly, however, the secondary deuterium kinetic isotope effects measured for 2,4-DNPC with E127A ($k_H/k_D = 1.08$) and with the wild-type enzyme ($k_H/k_D = 1.10$) are comparable, indicating a similar degree of oxocarbenium ion character at the respective deglycosylation transition states and probably a similar degree of C−O bond cleavage. Thus, the removal of the general base catalyst does not seem to have a significant effect upon the deglycosylation transition state structure at the anomeric centre.

pH Dependence of Cex E127A. As noted earlier, k_{cat} values for the wild-type enzyme are independent of pH as shown in Figure 4a. Interestingly, k_{cat} for E127A appears to be dependent upon a nonessential ionization of pK_a ~5.0 ± 0.1 in the basic limb of the pH profile. Instability of E127A at low pH values precluded a more accurate determination. Protonation of this group in the enzyme/substrate complex results in a faster deglycosylation step. By contrast, k_{cat}/K_m for hydrolysis of PNPC by wild-type enzyme is

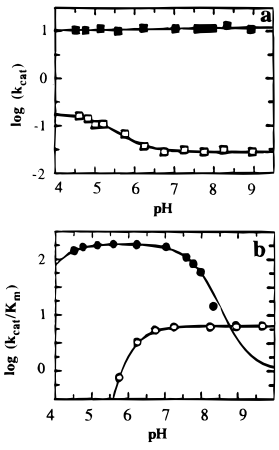


FIGURE 4: Dependence upon pH of kinetic parameters for the hydrolysis of cellobiosides by *C. fimi* exoglucanase (filled points) and its E127A mutant (open points).

dependent upon a group of pK_a = 7.7 in the free enzyme remaining protonated (Tull & Withers, 1994). No such loss in activity is observed at higher pH values in E127A, indicating that this group has been removed. This is exactly the behavior expected of an acid catalyst mutant. Similar changes in pH profiles upon removal of the active site acid catalysts have been seen in TEM-1 β-lactamase (Delaire *et al*., 1991), protein tyrosine phosphatase from *Yersinia enterocolitica* (Zhang *et al*., 1994), and Abg (Wang *et al*., 1995).

Values of k_{cat}/K_m for E127A, however, drop with pH below pH 7 according to a pK_a of approximately 6. This may reflect the protonation of the catalytic nucleophile, an event which would decrease its nucleophilicity. This large increase in pK_a relative to the wild-type enzyme where only a slight drop occurs below pH 5 is again likely a consequence of alterations in the hydrogen bonding network at the active site due to the removal of the acid/base catalyst. Inspection of the crystal structure of Cex (White *et al*., 1996) reveals that Glu 127 interacts with Asn 126, Gln 203, Trp 84, and a water molecule, all presumably involved in maintaining the ionization state of Glu 127 in the free enzyme. Mutation of Glu 127 to Ala would eliminate these hydrogen bonding interactions, and alter the local charge density within the active site. Thus, the shift in pK_a from approximately 4 to 6 is probably a consequence of these changes affecting the local environment, hence the pK_a of the catalytic nucleophile, Glu 233.

Elimination of Another Conserved Carboxylate: Asp 123. Several acidic residues in addition to the catalytic glutamates are conserved throughout family 10, these being Asp 43, Asp 123, Asp 170, Asp 235, and Asp 277 in Cex (MacLeod *et al*., 1994). Of particular interest is Asp 123 since its side chain is within hydrogen bonding distance of His 80, a conserved amino acid which interacts with the 3-hydroxyl of the proximal sugar in the bound substrate, and likely plays an important role in maintenance of active site charge. The consequences of mutation of this residue were different from those of the other mutants, the k_{cat} value for the hydrolysis of 2,4-DNPC by the mutant D123A being similar to that of the wild-type enzyme, whereas k_{cat} for hydrolysis of PNPC was reduced about 1500-fold (Table 2). This would indicate that the glycosylation step is now rate-limiting for PNPC and possibly also for 2,4-DNPC, there being a large leaving group dependence. Mutation of the equivalent residue D124 to N in XylA, a closely-related family 10 xylanase from *Streptomyces lividans*, also reduced PNPCase activity, but to a lesser extent (75-fold) (Moreau *et al*., 1994). This may reflect the ability of Asn but not Ala to participate in some of the hydrogen bonding interactions associated with the wild-type residue.

The k_{cat} value for the hydrolysis of PNPC by D123A was also found to be greater in the presence of both azide and thiocyanate, the rate enhancement with thiocyanate (data not shown) being about half that with azide. This might at first seem to be incompatible with the observation of rate-limiting glycosylation for this substrate. However, the reaction product under these conditions was found (by TLC) to be cellobiose and *not* cellobiosyl azide, indicating that the anion effect is on the first step as required by the kinetic studies. This clearly distinguishes the behavior of Asp123 from that of Glu127 or Glu233. It is not clear how azide or thiocyanate activates Cex D123A, though some form of catalytic rescue (Toney & Kirsch, 1989) seems most probable whereby the added anions serve to restore the local charge environment, allowing nearby residues such as His 80 to interact with the substrate.

Use of Anions To Probe the Functions of Conserved Carboxylates in Glycosidases. The fact that glycosyl azide products can be formed by Ala mutants at *both* the nucleophile and acid/base positions, but that such adducts are *not* formed with the Ala mutant of another conserved active site carboxylate, even though rate enhancements are observed, suggests an attractive strategy for the identification of the two key catalytic carboxylates in glycosidases. This would involve the identification of conserved carboxylates through sequence alignments, then generation of Ala mutants at each of these positions. Screening of these mutants with an activated chromogenic substrate in the presence and absence of azide would allow rapid identification of candidates. The reaction product formed in the presence of azide would then be identified either by ^{1}H-NMR or by chromatography and comparison with authentic standards. If the glycosyl azide has the same stereochemistry as the starting substrate, then the residue mutated is assigned as the acid/base catalyst. If it has the opposite stereochemistry, then the residue in question must be the catalytic nucleophile. Such an approach, which has now been shown to be feasible with Cex and with Abg (Wang *et al*., 1994, 1995) and in part with *E. coli* β-galactosidase (Huber & Chivers, 1993) and *B. subtilis* xylanase (S. Lawson, unpublished results), should be applicable to all retaining glycosidases and would allow relatively facile identification of these two important residues without the need for active site labeling and sequencing.

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