The Acid/Base Catalyst in the Exoglucanase/Xylanase from *Cellulomonas fimi* Is Glutamic Acid 127: Evidence from Detailed Kinetic Studies of Mutants†

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ABSTRACT: The exoglucanase/xylanase Cex from *Cellulomonas fimi* hydrolyzes β-1,4-glycosidic bonds with net retention of anomeric configuration, releasing the disaccharides β-cellobiose or β-xylobiose. It uses a double-displacement mechanism involving a glycosyl-enzyme intermediate which is formed and hydrolyzed with general acid/base catalytic assistance. Glu127 was proposed as the acid/base catalyst on the basis of sequence alignments, and mutants at this position were constructed in which the glutamic acid is replaced by alanine or glycine. The following kinetic analysis provides firm support for the assignment of Glu127 as the acid/base catalyst and suggests a more general strategy for identification of this residue in other glycosidases. Substrates which do not require protonic assistance for initial bond cleavage exhibit k_{cat}/K_m values similar to those of wild-type enzyme, whereas substrates which do require assistance have k_{cat}/K_m values over 6000-fold smaller. Thus rate constants for glycosylation are affected to different degrees by this substitution, depending upon their need for acid catalysis. The deglycosylation rate constant is decreased 200-fold by such substitution, due to the removal of general base catalytic assistance. In the presence of sodium azide a new product, β-cellobiosyl azide, is formed with these mutants whereas only cellobiose is formed with wild-type enzyme or the Glu127Asp mutant under similar conditions. Addition of azide results in very significant increases in k_{cat} values, ranging from 8-fold for 4″-nitrophenyl cellobioside to over 200-fold for 2″,4″-dinitrophenyl cellobioside, whereas k_{cat}/K_m values for these substrates remain essentially constant. No effects on rate upon azide addition are seen with substrates containing aglycons of poor leaving group ability. These results suggest that azide occupies a vacant anionic site created by removal of the acid/base catalyst and reacts rapidly with the glycosyl-enzyme intermediate, increasing the steady-state rate and forming the glycosyl azide product. The techniques employed in this study may be generally applicable to the identification of the acid/base catalyst in any cloned glycosidases belonging to sequence-related families.

The exoglucanase/xylanase Cex[1] from the bacterium *Cellulomonas fimi* hydrolyzes β-1,4-glycosidic bonds in cellulose and xylan, two residues in from the nonreducing terminus, with release of β-cellobiose or β-xylobiose (Withers *et al.*, 1986). Hydrolysis therefore occurs with retention of anomeric configuration, likely by a mechanism involving the formation and hydrolysis of a covalent α-D-glycopyranosyl-enzyme intermediate via oxocarbonium ion-like transition states (Sinnott, 1990; Tull & Withers, 1994). A number of amino acids must play important roles in binding and stabilizing these transition-state complexes, but two amino acids play particularly crucial roles. One functions as a catalytic nucleophile in formation of the intermediate while another functions as an acid/base catalyst.

The nucleophile in a retaining β-glycanase can be identified using an activated 2-deoxy-2-fluoro glycoside mechanism-based inactivator which functions by forming a highly stabilized intermediate. In particular, we have reported previously that the inactivation of Cex with 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside leads to the accumulation of the covalent 2-deoxy-2-fluoro-α-D-glucopyranosyl-enzyme intermediate in which the sugar is esterified to Glu233[2] (Tull *et al.*, 1991). Similar inactivation has been seen with 2″,4″-dinitrophenyl 2-deoxy-2-fluoro-β-cellobioside (Tull & Withers, 1994). The trapped intermediate is known to be catalytically competent because the inactivated enzyme can be reactivated by transglycosylation using cellobiose (Tull *et al.*, 1991). In addition, mutants of Cex modified at this position have been generated and analyzed, and their kinetic properties are fully consistent with Glu233 being the nucleophile (MacLeod *et al.*, unpublished observations).

No reliable strategies have, however, been devised for unequivocal labeling of the acid/base catalyst in a retaining glycanase. Group-specific reagents such as water-soluble

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[1] Abbreviations: Cex, *Cellulomonas fimi* exoglycanase; IPTG, isopropyl β-D-thiogalactopyranoside; PNPC, 4″-nitrophenyl β-cellobioside; PNPG, 4′-nitrophenyl β-D-glucopyranoside; 4-BrPC, 4″-bromophenyl β-cellobioside; 2,4-DNPC, 2″,4″-dinitrophenyl β-cellobioside; 2,4-DNPG, 2′,4′-dinitrophenyl β-D-glucopyranoside; 2F-DNPC, 2″,4″-dinitrophenyl 2-deoxy-2-fluoro-β-cellobioside; 2F-DNPG, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside; PEG, polyethylene glycol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; LB, Luria–Bertani medium.

[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.

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	a/b		n	
a -	NHVTKVADHFEGK_____	VASWDVVNEAFA -130	VDVRITELDIRM -	238
b -	DHINGVMAHYKGK_____	IVQWDVVNEAFA -	VDVAITELDIQ_ -	
c -	NHITTVMTHYKGK_____	IVEWDVANECMD -	VIVSFTEIDIRI -	
d -	NHITTVMQHYKGK_____	IYAWDVVNEIF_ -	KEIAVTELDI__ -	
e -	_____MKNYIGK_____	AFAWDVVNEAF_ -	_____ -	
f -	NHITTVMKQYKGK_____	LYAWDVVNEIF_ -	EEVAVTELDI__ -	
g -	SMIKNTFAALKSQYPNL_	DVYSYDVCNELFL -	LEVQITELDITC -	
h -	FYVKSVMGHFYSGKTGST_	LVYWDVCNETL_ -	FEVQITELDITN -	
i -	SYIHGVLDFVQTNYPGI__	IYAWDVVNE_IV -	LQIHITELDMH_ -	
j -	SYIKQVIEFCQKNYPGV__	VYCWDVVNEAIL -	LQIHITELNFEI -	
k -	KHIQTVVGRYKGK_____	VYAWDVVNEAID -	LEIHFTEIDISI -	
l -	QYIYDVVGRYKGK_____	VYAWDVVNEAID -	IEIHITELDMSL -	
m -	THITTVLDHFKTKYGAQNP	IIGWDVVNEVLD -	VEIQVTELDMNM -	
n -	THITTVLDHFKTKYGSQNP	IIGWDVVNEVLD -	VEIQVTELDMNM -	
o -	NHIKTVVERYK_____	DDVTSWDVVNEVID -	LDNQVTELDMSL -	
p -	NYIRAVVLRYK_____	DDIKSWDVVNEVIE -	LDNIITELDMSI -	
q -	EHIKTLCERYK_____	DVVYAWDVVNEAVE -	LEIHITELDISV -	
r -	QWIRDYCARYP_____	DTAMIDVVNEAVP -	KPIYISEYDIGD -	
s -	NHIDNVIGRYK_____	DDLAYFDIVNEPL_ -	_EVPMTELDVRI -	
t -	RHIDTVAAHFAGQ_____	VKSWDVVNEALF -	LKIKITELDVRL -	

FIGURE 1: Alignment of family F. The catalytic nucleophile in Cex is indicated by an "n". The putative acid/base catalyst is indicated by an "a/b". Conserved acidic residues are indicated in boldface. Only a portion of each polypeptide is shown. Accession numbers (Genbank or SWISS-PROT) are indicated in parentheses. (a) *C. fimi* Cex (L11080); (b) *Streptomyces lividans* XynA (M64551); (c) *Clostridium thermocellum* XynZ (M22624, P10478); (d) *Aspergillus kawachii* XynA (D14847); (e) *Thermoascus aurantiacus* XYN (P23360); (f) *Penicillium chrysogenum* Xyn (S31307); (g) *Ruminococcus flavefaciens* XynA (P29126); (h) *Butyrivibrio fibrisolvens* XynA (P23551); (i) *B. fibrisolvens* XynB (X61495, S55274); (j) *Caldocellum saccharolyticum* ORF4 (M34459); (k) thermophilic bacterial sp rt8.84 XynA (L18965); (l) *C. saccharolyticum CelB* (A43802, X13602); (m) *C. thermocellum* XynX (M67438); (n) *Thermoanaerobacter saccharolyticum* (strain B6A-RI) XylA (M97882); (o) *Bacillus* sp. strain C-125 XynA (D00087, P07528); (p) *Clostridium stercorarium* strain F9 (D12504); (q) *C. saccharolyticum* XynA (M34459); (r) *Pseudomonas fluorescens* subsp-cellulosa XynB (P23030); (s) *Cryptococcus albidus* XynA (JS0734); (t) *P. fluorescens* subsp-cellulosa XynA (X15429).

carbodiimides have been used to derivatize carboxylic acid residues with unusually high pK_a values; however, these frequently label at multiple sites. Affinity labels such as glycosyl epoxides have also proved unreliable in this regard (Legler, 1990). Direct investigation *via* X-ray crystallographic analysis of enzyme/substrate or enzyme/inhibitor complexes is a very convincing approach, but also very limited in its application. Although the catalytic domain of Cex has been crystallized and subjected to preliminary X-ray diffraction analysis (Bedarkar *et al.*, 1992), to date, the structure has not been solved. In the absence of a detailed three-dimensional structure for this enzyme, an alternative approach to identification of the acid/base catalyst, which should also be applicable to other enzymes, was sought.

β-1,4-Glycanases can be grouped into several families on the basis of similar amino acid sequences (Henrissat *et al.*, 1989; Beguin, 1990; Gilkes *et al.*, 1991; Henrissat, 1991). All members of a single family exhibit the same stereospecificity; *i.e.*, reaction proceeds with either retention or inversion of the configuration at the anomeric center (Gebler *et al.*, 1992b), suggesting that their active sites will have similar topologies. It is also very probable that catalytically important amino acids will be conserved within a family. As the number of enzymes assigned to a family increases, the number of strictly conserved amino acids decreases, thereby pinpointing potential catalytic residues. Aspartates and glutamates are the most likely candidates for these roles because they appear to be involved in the mechanisms of many glycanases and glycosidases [see Legler (1990) and Sinnott (1990) for recent reviews].

Cex belongs to family F of β-1,4-glycanases (Henrissat *et al.*, 1989). Currently, 20 enzymes can be assigned to the family. Four aspartates and two glutamates are conserved in all members of the family, corresponding to Asp43, Asp123, Glu127, Asp170, Glu233, and Asp277 in Cex (Shareck *et al.*, 1991; Figure 1, this study). One of these, Glu233, is the nucleophile in Cex, consistent with the notion of strictly conserved amino acids being important catalytically. Another of these residues, Glu127, is part of a highly conserved region, WDVVNEA. Within this region, the short consensus sequence NEX, where X is a small hydrophobic residue, occurs in a large number of glycanases around a completely conserved glutamate. This has been suggested previously (Baird *et al.*, 1990) to play an important role based upon alignments of sequences of a number of cellulases. It therefore seems likely that this residue functions as the acid/base catalyst in Cex and other glycanases. This paper describes the isolation and characterization of mutants of Cex in which Glu127 is replaced by Asp, Ala, and Gly. The properties of the mutants are consistent with Glu127 being the acid/base catalyst in Cex.

EXPERIMENTAL PROCEDURES

Reagents. Growth media components were from Difco. Restriction endonucleases, polymerases, and nucleotides were from Pharmacia and BRL. Radionucleotides were from New England Nuclear Corp. IPTG, PNPC, and PNPG were from Sigma. 4-BrPC and 2,4-DNPC (Tull & Withers, 1994) and 2F-DNPC (McCarter *et al.*, 1993) were synthesized as described previously. All buffer chemicals were obtained from Sigma and BDH.

Mutagenesis. The *cex* gene (1.8 kb), encoding the exoglucanase/xylanase (Cex) from *C. fimi*, was subcloned from pUC12-1.1Cex(PTIS) (O'Neill *et al.*, 1986) into pTZ18R (Mead *et al.*, 1986) and then transformed (Hanahan, 1983) into *Escherichia coli* RZ1032. Single-stranded DNA was obtained as follows: Single, overnight colonies were inoculated to TYP medium (Withers *et al.*, 1992) containing 100 $\mu g \cdot mL^{-1}$ ampicillin and 10^9 PFU$\cdot mL^{-1}$ M13K07 helper phage. Kanamycin was added to 70 $\mu g \cdot mL^{-1}$ after 1 h of incubation. Cultures were grown overnight at 37 °C at 225 rpm. Cells were removed by centrifugation, and phagemid were precipitated with 1.7 M ammonium acetate and 12% (w/v) PEG-6000. Single-stranded DNA was isolated from the phagemid

as described previously (Kristensen *et al.*, 1987). Site-directed *in vitro* mutation (Kunkel *et al.*, 1987) at Glu127 used 25mer synthetic oligonucleotide primers: 5′P-AC GTC GTC AAC XXX GCG TTC GCC GA-3′. The positions of mismatches are indicated by the underlined X's; for E127D, XXX = GAC; for E127A, XXX = GCG; for E127G, XXX = GGC. Following *in vitro* mutation, the plasmids were transformed to *E. coli* JM101. Initial screening of Cex mutants was on LB agar containing 100 $\mu g \cdot mL^{-1}$ ampicillin (LB/amp) and 100 μM methylumbelliferyl cellobioside. Nonfluorescing colonies, indicating loss of exoglucanase activity, were isolated, and plasmid DNA was prepared for sequencing. Sequencing was performed as described previously (Withers *et al.*, 1992). An 800 base pair region surrounding the mutation of interest was sequenced to confirm that only the desired mutation was present. The sequenced, mutant cassettes were subcloned back to pUC12-1.1Cex(PTIS). The mutant plasmids were transformed into *E. coli* JM101 for expression.

Protein Production and Purification. Two-liter cultures in shake flasks were grown overnight in LB/amp. At A_{600} = 1.0, IPTG was added to 0.1 mM. The cells were collected by centrifugation and resuspended in 75 mL of 50 mM phosphate buffer, pH 7.0 (phosphate buffer). Cells were passed twice though a french pressure cell; phenylmethanesulfonyl fluoride was added to 0.5 mM, pepstatin A to 1 μM, and EDTA to 1 mM. Following clarification of the cell extract by centrifugation at 40000*g* for 30 min, streptomycin sulfate was added to 1.5% and the mixture stirred at 4 °C overnight. The extract was centrifuged again at 40000*g* for 30 min.

The supernatant was stirred with 50 g of CF-1 cellulose (Sigma) in phosphate buffer for 3 h at 4 °C. The cellulose was washed twice with 500 mL of 1 M NaCl in phosphate buffer and twice with 500 mL of phosphate buffer. The cellulose was packed into a column (5 cm × 25 cm), and adsorbed polypeptides were eluted with distilled water, flow rate 1 $mL \cdot min^{-1}$. The absorbance of the eluate was measured continuously at 280 nm. Peak fractions were pooled and centrifuged at 40000*g* for 30 min. The supernatant was concentrated by diafiltration through an Amicon PM10 membrane and then analyzed by SDS–PAGE (Laemmli, 1970). The solution was passed through a 0.22-μm filter (Millipore) and then stored in phosphate buffer at 4 °C.

Enzyme Kinetics. Michaelis–Menten parameters for various substrates were determined by measuring the rate of release of *p*-nitrophenol, dinitrophenol, or *p*-bromophenol spectrophotometrically. Reactions were carried out at 37 °C in phosphate buffer at substrate concentrations ranging between $0.14 \times K_m$ and $7 \times K_m$. Where indicated, sodium azide was included in the reactions at the concentrations shown. The extinction coefficients used and wavelengths monitored for each substrate were as follows: PNPC, λ = 400 nm, ϵ = 7280 $M^{-1}\ cm^{-1}$; 2,4-DNPC, λ = 400 nm, ϵ = 10 900 $M^{-1}\ cm^{-1}$; 4-BrPC, λ = 288 nm, ϵ = 1120 $M^{-1}\ cm^{-1}$.

E127A was inactivated with 2F-DNPC as described previously for inactivation of the native enzyme by 2F-DNPG (Tull *et al.*, 1991) with the following changes. Residual enzyme activity was monitored at various times by transferring 10-μL aliquots of inactivation mixture into a final volume of 500 μL of phosphate buffer containing 100 μM 2,4-DNPC and 60 mM sodium azide.

Synthesis and Characterization of β-Cellobiosyl Azide and β-Glucosyl Azide. Syntheses of β-cellobiosyl azide and β-glucosyl azide to be used as standards were carried out essentially as described previously (Micheel & Klemer, 1961). Samples were dissolved in D_2O, freeze-dried several times, and finally dissolved in D_2O. ^{1}H-NMR spectra were recorded on a 400-MHz Bruker NMR and the following spectra obtained: β-Cellobiosyl azide: ^{1}H-NMR (400 MHz, D_2O) δ 4.47 (d, $J_{1,2}$ 7.8 Hz, H-1), 3.89 (m, 3 H), 3.65 (m, 4 H), 3.35 (m, 6 H). β-Glucosyl azide: ^{1}H-NMR (400 MHz, D_2O) δ ~4.7 (d, $J_{1,2}$ 9.0 Hz, H-1), 3.89 (dd, 1 H, $J_{6,6'}$ 12.4 Hz, $J_{6,5}$ 2.2 Hz, H-6), 3.71 (dd, 1 H, $J_{6',6}$ 12.4 Hz, $J_{6',5}$ 5.6 Hz, H-6′), 3.50 (m, 2 H, H-3, 5), 3.40 (t, 1 H, $J_{4,3}$ 9.2 Hz, $J_{4,5}$ 9.8 Hz, H-4), 3,24 (t, 1 H, $J_{2,1}$ 9.0 Hz, $J_{2,3}$ 8.9 Hz, H-2).

Thin-layer chromatograms on 60 F254 silica gel aluminum plates (E. Merck) were run in 7:2:1 (v/v/v) ethyl acetate/methanol/water and developed with 10% H_2SO_4 in methanol. The R_f values for β-cellobiosyl azide and β-glucosyl azide were 0.44 and 0.64, respectively. Products were characterized from reactions containing 100 $\mu g \cdot mL^{-1}$ enzyme and 6 mM substrate in phosphate buffer. Sodium azide was included at 60 mM where indicated.

Table 1: Kinetic Parameters for Hydrolysis of Various Substrates by Cex and E127 Mutants

enzyme	substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($min^{-1}\ mM^{-1}$)
native Cex	DNPC	419	0.06	6983
	PNPC	677	0.53	1278
	PBrPC	255	2.0	128
E127A	DNPC	2.4	0.0003	7742
	PNPC	2.3	0.025	92
	PBrPC	0.04	1.9	0.02
E127G	DNPC	2.1	0.0004	5526
	PNPC	2.0	0.029	69
E127D	DNPC	1.6	0.0017	941
	PNPC	3.3	1.74	2

RESULTS

Cloning, Purification, and Physical Characterization of Mutants. Following site-directed mutagenesis and transformation to *E. coli* JM101, initial screening for Cex mutants was on LB agar containing 100 $\mu g \cdot mL^{-1}$ ampicillin (LB/amp) and 100 μM methylumbelliferyl cellobioside. Approximately 50% of the colonies were nonfluorescing, indicating loss of exoglucanase activity. DNA sequencing revealed approximately 80% of the nonfluorescing colonies contained only the mutation of interest. The three mutants obtained, E127D, E127A, and E127G, were expressed at approximately the same levels as the wild type. The preparations obtained by affinity chromatography on cellulose had the same M_rs as wild-type Cex and were >95% homogeneous when analyzed by SDS–PAGE (not shown). No differences in behavior were observed during the purification procedure.

Kinetic Analysis. Michaelis–Menten parameters were determined for each enzyme with two different aryl cellobioside substrates, 4″-nitrophenyl β-D-cellobioside (PNPC) and 2″,4″-dinitrophenyl β-D-cellobioside (2,4-DNPC). In addition, E127A was assayed with 4″-bromophenyl β-D-cellobioside (4-BrPC). The parameters determined for E127 mutants are shown in Table 1. The cellobioside substrates were found to have K_m values in the low micromolar region, necessitating great care in monitoring of initial reaction rates.

Kinetic parameters for the inactivation of E127A by 2″,4″-dinitrophenyl 2-deoxy-2-fluoro-β-D-cellobioside (2F-DNPC) were determined as described in the Experimental Procedures. Difficulties were encountered both with the high inactivation rate (therefore difficulty in measurement of maximal rates) and with the insolubility of the inactivator at the higher concentrations which would be required in order to achieve saturation. These problems precluded determination of the individual kinetic parameters k_i and K_i, but a reliable value of k_i/K_i = 1.6 $min^{-1}\ mM^{-1}$ was obtained from the slope of the

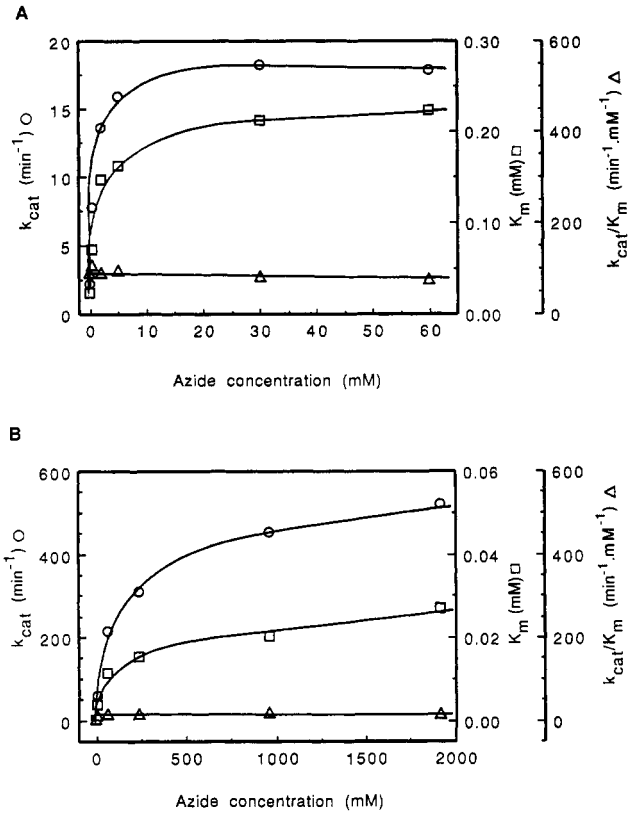


FIGURE 2: Kinetic parameters for hydrolysis of PNPC (panel A) and 2,4-DNPC (panel B) by Cex E127A in the presence of various concentrations of sodium azide.

reciprocal plot. For the wild-type enzyme, a value of k_i/K_i = 0.61 min^{-1} mM^{-1} was obtained (Tull & Withers, 1994).

Effects of Sodium Azide on Products and Reaction Rates. Thin-layer chromatographic analysis of reaction mixtures containing 2,4-DNPC revealed that Cex produced only the expected phenol plus cellobiose, both in the absence *and* in the presence of the competitive nucleophile sodium azide. With E127A and E127G the reaction products differed in the absence and presence of azide. In the absence of azide, only cellobiose plus phenol was observed in each case, while in its presence a different sugar product was formed. This compound comigrated on TLC with β-cellobiosyl azide obtained by chemical synthesis. Further, analysis by ^{1}H-NMR revealed an identical spectrum to that obtained from the chemically synthesized compound, providing confirmation of the β-stereochemistry.

In the case of the E127A and E127G, kinetic parameters for PNPC and 2,4-DNPC were found to be substantially affected by the presence of azide. Maximal increases in k_{cat} ranged from 8-fold for PNPC in the presence of 60 mM azide to more than 200-fold for 2,4-DNPC at 2 M azide, as shown in Figure 2. In each case, values of K_m also increased as the azide concentration was raised, while values of k_{cat}/K_m remained approximately constant. No such increases in k_{cat} were seen for 4-BrPC as azide concentration was increased (data not shown).

DISCUSSION

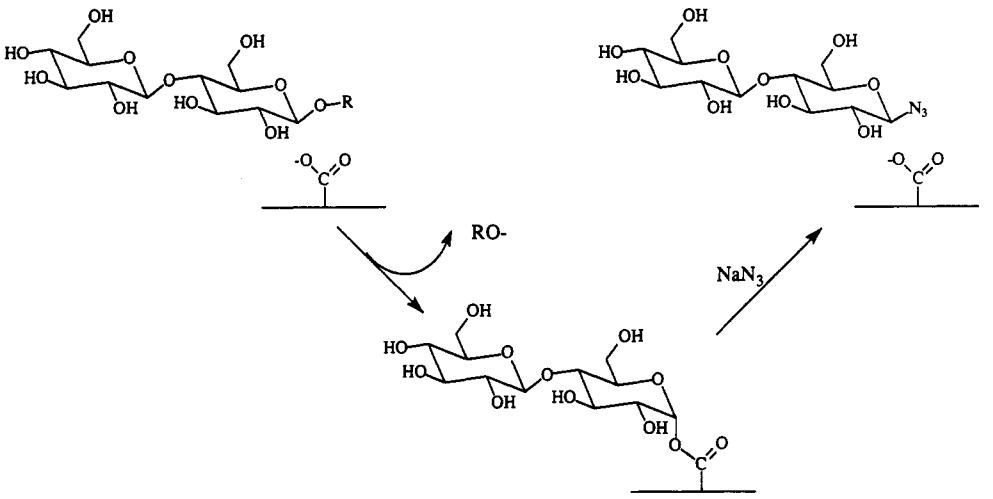
The catalytic mechanism of retaining glycosidases such as Cex involves a two-step process: formation and hydrolysis of a glycosyl-enzyme intermediate [see Scheme 1 in Tull and Withers (1994), preceding paper in this issue]. In both steps a proton transfer occurs, to and from an oxygen atom in an equatorial position at the anomeric center. It is therefore likely that a single amino acid residue (the acid/base catalyst) is responsible for both proton-transfer steps. In the first step a proton transfer occurs from this residue to the glycosidic oxygen as the glycosidic linkage is cleaved, thereby facilitating bond cleavage through stabilization of the leaving group. In the second step, this same residue functions as a general base catalyst, removing a proton from water in a concerted process as the water attacks the anomeric center of the glycosyl-enzyme intermediate. It is therefore clear that modification of this acid/base residue will affect the rates of *both* steps. However, the extent to which each step is affected is not necessarily equivalent. The effect of such a modification to the acid/base catalyst on the rate of the second step, deglycosylation, will necessarily be identical for all substrates containing the same sugar residue. However, the effects on the first step (glycosylation) will depend upon the leaving group ability of the aglycone. Those of high pK_a, and, therefore, poor leaving group ability, will be affected most, while those of low pK_a, which need little or no protonic assistance for departure, will be affected very little, if at all. These expectations are indeed borne out for the E127 mutants of Cex, as discussed below:

As can be seen from the kinetic data in Table 1, the effect of mutation of Glu127 on k_{cat} values of nitrophenyl cellobiosides (PNPC, pK_a of phenolic leaving group = 7.18, and 2,4-DNPC, pK_a of phenolic leaving group = 3.96) is to decrease them approximately 200- to 300-fold. Previous studies (Tull & Withers, 1994) have shown that deglycosylation is the rate-determining step for these substrates with wild-type enzyme. On the basis of the data in Table 1 it seems likely that deglycosylation is also the rate-determining step for these mutants with the same nitrophenyl cellobioside substrates, as follows. First, essentially the same k_{cat} values are seen for two substrates with aglycons of quite different leaving group ability, as required if the second step is rate-determining. Second, K_m values are extremely low, also, as would indeed be expected if the second step were rate-limiting, due to accumulation of the glycosyl-enzyme intermediate. Removal of the carboxyl group of E127 therefore results in a slowing of the deglycosylation step by 200-fold, with no change in rate-determining step. This therefore provides a fairly direct measure of the contribution of general base catalytic assistance to catalysis.

A good measure of the effect of this mutation on the glycosylation step for these substrates is obtained by study of k_{cat}/K_m values which reflect the first irreversible step, most probably the formation of the glycosyl-enzyme intermediate. Essentially no reduction in k_{cat}/K_m is seen with 2,4-DNPC (with the exception of the E127D mutant) while a 10–20-fold reduction is seen for PNPC. An additional measure of the effect of this mutation on the glycosylation step resides in the finding that the rate of inactivation of E127A by 2F-DNPC (k_i/K_i = 1.6 min^{-1}mM^{-1}) is at *least* as fast as that of the wild-type enzyme (k_i/K_i = 0.61 min^{-1} mM^{-1}). Since the inactivation involves simply the formation of a 2-deoxy-2-fluoroglycosyl-enzyme intermediate, it is clear that mutation at E127 does not slow down the glycosylation step of substrates with very good leaving groups.

By contrast, the k_{cat} value for 4-BrPC (pK_a of phenolic leaving group = 9.34) is lowered some 6400-fold upon mutation of Glu127 to Ala. The rate-determining step for this substrate with wild-type enzyme is formation of the glycosyl-enzyme, and the fact that the K_m value for the mutant is similar to that of the wild type would suggest that this step remains rate-limiting with the mutant. The 6400-fold rate reduction

Scheme 1



therefore represents the effect upon the glycosylation step for this substrate, an effect which is significantly greater than that seen on the deglycosylation step and much greater than that seen on the glycosylation step for DNPC. In agreement with this a similar reduction in k_{cat}/K_m (6100-fold) is also observed with 4-BrPC. This is exactly the behavior expected if E127 is indeed the acid/base catalyst, since removal of protonic assistance should cause large reductions in the glycosylation rate for substrates with poor leaving groups which need protonic assistance (e.g., 4-BrPC), but only minimal reductions for substrates with good leaving groups (e.g., 2,4-DNPC). Effects on the deglycosylation step are necessarily identical for all substrates.

Not only does the mutation of Glu127 to Ala or Gly ensure that the deglycosylation step is firmly rate-limiting for substrates with good leaving groups, it also presumably generates a small cavity in the active site, possibly capable of binding an anion. If indeed Glu127 is the acid/base catalyst, this cavity should be close to the β-face of the substrate. Small anions of high nucleophilicity, particularly those such as azide which react preferentially with cationic transition states, might bind at this site and react in place of water without any need for general base catalysis. Such a mechanism of activation by azide requires, of course, that the reaction product be the corresponding β-glycosyl azide (Scheme 1). Such indeed was shown to be the case by both TLC analysis of reaction mixtures using authentic β-cellobiosyl azide as reference material, and by NMR analysis of reaction products.[3] The fact that *only* β-cellobiosyl azide was observed confirms that attack of azide is taking place *only* from the top face, a result consistent with a location of the acid/base catalyst on this same face.

Anions such as azide should also therefore significantly increase the steady-state rate for substrates whose rate-determining step is deglycosylation, as indeed has proved to be the case with the E127A and E127G mutants. This has been studied in some detail with the alanine mutant. As seen in Figure 2A, the steady-state rate for PNPC increased by over 8-fold as the azide concentration was increased up to 60 mM and then leveled off. Initially, it was assumed that this leveling off of rates was due to saturation of a binding site for azide. However, this proved not to be the case since much more dramatic increases in k_{cat} of over 200-fold were seen with 2,4-DNPC (Figure 2B), surpassing the rate of the wild-type enzyme, but such rate increases required much higher concentrations of azide, up to 2 M. Since the same step is being monitored in each case, some other explanation is necessary. The most likely reason for this leveling is that as azide is added, the deglycosylation rate continues to increase until it surpasses the glycosylation rate for that particular substrate. The limiting rate observed in each case therefore presumably reflects the rate of the glycosylation step for each substrate. Since the glycosylation step is much faster for 2,4-DNPC than for PNPC (*vide supra*), the limiting rate in the presence of azide is much larger in the former case than the latter.

In contrast, no increase in k_{cat} for 4-BrPC, a substrate for which glycosylation *is* the rate-limiting step, was observed upon addition of azide (not shown). This finding is entirely reasonable since the rate-determining step for this substrate has previously been shown to be the formation of the glycosyl-enzyme; thus, increasing the rate of the deglycosylation step will not affect the steady-state rate. Interestingly, no increases in k_{cat} with increasing azide were seen for the wild-type enzyme, or with E127D. Presumably, this is due to the presence of the anionic enzymic carboxylate on the β-face, which prohibits access by the azide nucleophile through electrostatic repulsion.

Dramatic increases in K_m values with increasing azide concentration are also observed for each substrate (Figure 2), essentially paralleling the effects on k_{cat}. Such increases are a consequence of the relatively decreased extent of accumulation of the intermediate as the deglycosylation step speeds up. The net result of this is that values of k_{cat}/K_m remain essentially constant across the range. Such a finding is important as it confirms that the effect of the azide is primarily on the deglycosylation step, and not on glycosylation. It is, however, possible that azide could have effects on the first step since the azide could in principle bind to the site created and help to stabilize an active structure, either conformationally or in terms of active site group pK_a values.

In summary, detailed kinetic analysis of mutants of Cex selected on the basis of sequence alignments has provided strong evidence for the identity of E127 as the acid/base catalyst of this enzyme. The techniques employed may be generally applicable to any cloned glycosidases belonging to sequence-related families (Henrissat, 1991). The approach would involve generation of alanine or glycine mutants of the conserved glutamic and aspartic acids, and then screening of these mutants for the generation of new products of hydrolysis

[3] During the preparation of this paper we became aware of similar observations on mutants of *E. coli* β-galactosidase modified at position Glu461 (R. E. Huber, personal communication).

in the presence of azide. Positive mutants should then be subjected to the following kinetic analysis. First, k_{cat} and K_m values should be measured for a pair of substrates, one of which requires acid catalysis and one of which does not. Values of k_{cat}/K_m for these two substrates should be vastly different, that for the activated substrate being much higher than that for the other. Further, values of k_{cat} and K_m for highly activated substrates with these mutants should increase dramatically with azide concentration and level off at a value dependent on the leaving group ability of the aglycon. This sort of approach could facilitate identification of the acid/base catalyst in other glycosidases, and quite possibly other glycosyl transferases. Indeed, preliminary results on such mutants of two other glycosidases, *Agrobacterium* β-glucosidase and *Bacillus subtilis* xylanase, plus a reinterpretation of the results of Cupples *et al.* (1990) on mutants of *E. coli* β-galactosidase (Gebler *et al.*, 1992a) would indicate that this approach is indeed a truly general one.

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