Identification of the Acid/Base Catalyst in *Agrobacterium faecalis* β-Glucosidase by Kinetic Analysis of Mutants†

Q. Wang,‡ D. Trimbur,§,|| R. Graham,§,⊥ R. A. J. Warren,§ and S. G. Withers*,‡

Protein Engineering Network of Centres of Excellence of Canada and Departments of Chemistry and Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z1

Received May 25, 1995; Revised Manuscript Received August 9, 1995⊗

ABSTRACT: The catalytic mechanism of the retaining β-glucosidase (Abg) from *Agrobacterium faecalis* involves a double-displacement process in which an α-glucosyl−enzyme intermediate is formed with general acid catalytic assistance and then hydrolyzed with general base assistance. Glu170 was identified as an important residue, possibly the acid/base catalyst, on the basis of sequence alignments. This glutamate is conserved in almost all enzymes in family 1 of glycoside hydrolases. Detailed pre-steady-state and steady-state kinetic analyses of the mutant E170G suggested very strongly that Glu170 is the acid/base catalyst. First, k_{cat} values were invariant with pH over the range of 5.0−9.0. Secondly, rates of formation of the glycosyl−enzyme, calculated from k_{cat}/K_m and k_2, were similar to those of wild-type enzyme for substrates not requiring protonic assistance but dramatically reduced for those needing acid catalysis. Thirdly, addition of azide as a competitive nucleophile increased k_{cat} values 100−300-fold for substrates whose rate-limiting step is deglycosylation, yielding β-glucosyl azide, but had no effect on the wild-type enzyme. Other anionic nucleophiles had similar, but less dramatic effects. Previous results [Gebler, J. C., et al. (1995) *34,* 14547−14553] had indicated that Tyr298 is important for catalysis. The kinetic consequences of the mutations in the double mutant E170G-Y298F are additive, resulting in a 10^6-fold reduction in k_{cat} values and allowing the accumulation of a stable ($t_{1/2}$ > 7 h) glucosyl−enzyme intermediate. Thus, Glu170 and Tyr298 function independently, and a possible role for Tyr298 in modulating the pK_a of the catalytic nucleophile is proposed.

The β-glucosidase (Abg)[1] from *Agrobacterium faecalis* hydrolyzes β-glucosides with net retention of anomeric configuration (Day & Withers, 1986), likely via a double-displacement mechanism involving a covalent α-D-glucopyranosyl−enzyme intermediate [Sinnott, 1990; Withers & Street, 1988; Kempton & Withers, 1992; see Scheme 1 in the preceding paper by Gebler et al. (1995)]. Two active site amino acid residues play key roles in this process. One serves as a nucleophile, attacking the anomeric centre of the glucoside substrate and displacing the leaving group to form a covalent α-D-glucopyranosyl−enzyme intermediate. This residue was identified as Glu358 by labeling with the mechanism-based inactivator 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside (Withers et al., 1990). The other key residue serves as a general acid/general base catalyst, providing protonic assistance in the first step for the departure of the leaving group and presumably also serving as the general base by deprotonating the water as it attacks the glycosyl−enzyme intermediate.

In all cases in which these catalytic groups have been reliably identified to date, they are the carboxylic side chains of glutamic or aspartic acid residues (Legler, 1990; Sinnott, 1990). Possible candidates for the role of acid/base catalyst can therefore be identified by searching for conserved glutamic and aspartic acid residues through sequence alignments of related enzymes. Abg belongs to family 1 in the recent classification of glycosidases on the basis of sequence similarities (Henrissat, 1991; Henrissat & Bairoch, 1993). This family now contains at least 24 members. Several glutamates and two aspartates are conserved, or essentially conserved, in all members of the family (Figure 1). Of particular interest is Glu170 (Abg numbering), which resides within a highly conserved region characterized by the consensus sequence TXNEX, where X is a hydrophobic residue. The common occurrence of such a sequence in cellulases was noted previously (Baird et al., 1990). The glutamate within the sequence in an enzyme from family 10 was shown, through kinetic analysis of mutants, to function as an acid/base catalyst (MacLeod et al., 1994); X-ray crystallography showed the glutamate to lie within the active site of the enzyme (White et al., 1994). Tyr298 is conserved throughout family 1. This residue is also crucial to catalytic efficiency, perhaps playing a role in acid catalysis (Gebler et al., 1995). Therefore, it was of interest to examine properties of Abg mutated at Glu170 and Tyr298.

† Financial support from the Natural Sciences and Engineering Research Council of Canada and the Protein Engineering Network of Centres of Excellence of Canada.

* To whom correspondence should be addressed.

‡ Department of Chemistry.

§ Department of Microbiology.

|| Present addresses: Genencor, 180 Kimball Way, South San Francisco, CA 94080.

⊥ Present address: Inex Pharmaceuticals, 1779 West 75th Avenue, Vancouver, BC, Canada V6P 6P2.

⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1995.

[1] Abbreviations: Abg, *Agrobacterium faecalis* β-glucosidase; 2,4-DNPG, 2′,4′-dinitrophenyl β-D-glucopyranoside; 3,4-DNPG, 3′,4′-dinitrophenyl β-D-glucopyranoside; 2,5-DNPG, 2′,5′-dinitrophenyl β-D-glucopyranoside; 4-Cl-2NPG, 4-chloro-2-nitrophenyl β-D-glucopyranoside; ONPG, *o*-nitrophenyl β-D-glucopyranoside; PNPG, *p*-nitrophenyl β-D-glucopyranoside; IPTG, isopropyl β-D-thioglucopyranoside; DNP-2FGlu, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside; PG, phenyl β-D-glucopyranoside.

0006-2960/95/0434-14554$09.00/0

FIGURE 1: Sequence alignment in the β-glycosidase family. Only portions of each polypeptide are shown. Accession numbers (Genbank or SWISS-PROT) are indicated in parentheses. (a) *Clostridium thermocellum* β-glucosidase (S17215); (b) *Agrobacterium sp* β-glucosidase (A28673); (c) *Bacillus polymyxa* β-glucosidase A (JW0037); (d) *Thermotoga maritima* β-glucosidase (S34570); (e) *Bacillus polymyxa* β-glucosidase B (JW0038); (f, g, h) *rabbit* β-glycosidase complex (S01169); (i) *White Clover* β-glucosidase precursor (S16581); (j) *White mustard* thioglucosidase (S19149); (k) *Lactobacillus casei* 6-phospho-β-galactosidase (A29897); (l) *Caldocellum saccharolyticum* β-glucosidase (S03813); (m) *Staphylococcus aureus* β-galactosidase (A27233); (n) *Sulfolobus solfataricus* β-galactosidase (S06762); (o, p, q) *rat* β-galactosidase/glycosylceramidase precusor (JS0610); (r) *Streptomyces rochei* β-glucosidase (S35958); (s) *Microbispora bispora* β-glucosidase, BglB (A48949); (t) *Cassava* β-glucosidase (S23940); (v) *Erwinia Chrysanthemi* ArbB=phospho-β-glucosidase (C42603); (w) *Escherichia coli* phospho-β-glucosidase (S27553); (x) *Escherichia coli* phospho-β-glucosidase=ascB (C44070); (y) *rape* myrosinase (S26149). Note: "o" and "f" are domains of the proteins with no catalytic activity.

This paper describes the generation and purification of a mutant of Abg in which Glu170 is replaced by Gly as well as a double mutant involving the additional replacement of Tyr298 by Phe. It also describes the detailed kinetic analysis of these mutants by both steady-state and pre-steady-state techniques according to an extended version of a recently described protocol (MacLeod et al., 1994), thereby providing strong evidence for the normal role of Glu170 as an acid/base catalyst.

EXPERIMENTAL PROCEDURES

Reagents. Growth media components were obtained from Difco. Restriction endonucleases, polymerases, and nucleotides were from Pharmacia and BRL. Radionucleotide was from New England Nuclear Corp. 2,4-DNPG, 3,4-DNPG, 2,5-DNPG, 4-Cl-2NPG, and ONPG were synthesized according to the published procedures (Kempton & Withers, 1992). PNPG and all buffer materials and other chemicals were obtained from Sigma and Aldrich Chemical Co.

Mutagenesis. pTZ18R::abg was mutated as described previously (Trimbur et al., 1992). The specific mutations E170G and Y298F were obtained individually using the oligonucleotide primers: pGCGACCTTCAACGGGCCTTGGTGCGCG and pGCCTGAATTATTNCACGCCGATGCG, respectively (the underlines show the mutated codons, and the N indicates the position where all four nucleotides were used in the second primer). The Y298F mutant was identified by single-track sequencing; both mutations were confirmed by sequencing the genes. The double mutant was obtained by combining appropriate restriction fragments bearing the individual mutations. Mutant *abg* genes were subcloned into plasmid pTug10N (Graham et al., 1994) and transformed into *Escherichia coli* strain R1360 (Trimbur et al., 1992) for protein production. Expression levels of mutant proteins were monitored by SDS−PAGE (Laemmli, 1970) and by kinetic assay with 2,4-DNPG.

Protein Production and Purification. Cultures were grown at 27 °C on TYP (16 g/L tryptone, 16 g/L yeast extract, 5

g/L NaCl, 2.5 g/L K_2HPO_4) medium containing 100 μg/mL carbenicillin (Gemini). IPTG was added at mid-log phase and incubation continued overnight. Cells were collected by centrifugation and resuspended in 80 mL of 25 mM sodium phosphate buffer (pH 7.00) containing 2 mM EDTA. Cells were passed twice through a French pressure cell. Following clarification of the cell extract by centrifugation at 14000*g* for 20 min, streptomycin sulfate was added to 1.5% (w/v) and the mixture stirred at 4 °C for 4−6 h. The extract was centrifuged again at 14000*g* for 30 min.

The mutant polypeptides were purified essentially according to the protocol used for the wild type recombinant *Agrobacterium* β-glucosidase (Kempton & Withers., 1992) except that the activity of the E170G mutant was determined by assay with 2,4-DNPG in the presence of azide. The purity of the mutants was assessed by polyacrylamide gel electrophoresis. Circular dichroism spectra were obtained for the E170G and E170G-Y298F mutants as well as wild-type Abg at 50 °C in 50 mM sodium phosphate buffer, pH 7.0. The thermal stabilities of the proteins were assessed by monitoring changes at 222 nm under these conditions.

Inactivation, Active-Site Titration, and Stoichiometry of Incorporation of Inactivator. The active sites of the mutants were titrated with DNP-2FGlu as titrant for the E170G mutant and both 2,4-DNPG and PNPG as titrants for the E170G−Y298Y mutant, as follows: an aliquot of the Glu170Gly mutant (6 μL of 5.0 mg/mL) was added to 0.58 mM DNP-2FGlu in 50 mM sodium phosphate buffer pH, 7.0, to give a total volume of 250 μL; E170G-Y298F (50 μL of 3.8 mg /mL) was added to DNPG (2 mM) or PNPG (3 mM) in 50 mM sodium phosphate buffer, pH, 7.0, to give a total volume of 200 μL. In all cases titrations were performed in 1 cm microcuvettes at 37 °C in a thermostated spectrophotometer. The release of nitrophenolate after the addition of an excess of titrant to a known quantity of mutant enzyme was monitored at 400 nm, essentially as described previously (Street et al., 1992). The concentration of nitrophenolate released (thus of active enzyme) was calculated using extinction coefficients of 10.9 and 7.28 mM^{-1} cm^{-1} for dinitrophenolate and nitrophenolate, respectively.

The inactivation of E170G with DNP-2FGlu was analyzed kinetically essentially as described previously (Street et al., 1992). Concentrations of DNP-2FGlu employed were 0.022, 0.040, 0.11, 0.14, 0.36, and 0.72 mM. Values of K_i and k_i were calculated from the pseudo-first-order rate constants for inactivation at each inhibitor concentration using the program Grafit (Leatherbarrow, 1990) as described previously (Street et al., 1992). Electrospray mass spectra were obtained with a PE/SCIEX API III Ion Spray LC/MS system. Samples of labeled and unlabeled enzyme (~10 μg) were injected into the mass spectrometer via a microbore HPLC system equipped with a polystyrene−divinylbenzene reverse-phase column (5 μm, 300 Å, 1 × 50 mm) using the water/acetonitrile solvent system, and then 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.

Steady-State Kinetic Studies. Rates of enzyme-catalyzed hydrolysis were determined by incubating the appropriate concentration of substrate in 50 mM sodium phosphate buffer, pH 7.0, containing 0.1% BSA at 37 °C in a 1 cm cuvette located in a thermostated spectrophotometer. Reaction was initiated by the addition of enzyme from a syringe and the reaction was monitored at the appropriate wavelength: 2,4-DNPG, 400 nm, $\Delta\epsilon$ = 10.9 mM^{-1} cm^{-1}; 2,5-DNPG, 440 nm, $\Delta\epsilon$ = 4.29 mM^{-1} cm^{-1}; 3,4-DNPG, 400 nm, $\Delta\epsilon$ = 11.05 mM^{-1} cm^{-1}; 4-Cl-2NPG, 425 nm, $\Delta\epsilon$ = 3.55 mM^{-1} cm^{-1}; PNPG, 400 nm, $\Delta\epsilon$ = 7.28 mM^{-1} cm^{-1}; ONPG, 400 nm, $\Delta\epsilon$ = 2.17 mM^{-1} cm^{-1}. Rates were determined at 5−7 different substrate concentrations ranging, where possible, from approximately 0.15 to 7 times the value of K_m. Values of K_m and k_{cat} were determined from those data by means of a nonlinear regression analysis using the program GraFit (Leatherbarrow, 1990). Very low values of K_m were obtained in some cases, necessitating particular care in monitoring of initial reaction rates. The hydrolysis of phenyl β-D-glucopyranoside by the E170G mutant was extremely slow; therefore, the rate was determined by hydrolysis of phenyl β-D-glucopyranoside (8.8 and 22.3 mM) with 15 mg/mL of the mutant in 50 mM sodium phosphate buffer, pH 7.0, for 3 days, using a coupled assay (Kunst et al., 1984) to quantitate glucose released. Careful control experiments monitored both enzyme stability and spontaneous hydrolysis of the substrate.

Buffers used for pH-dependence studies were 50 mM MES/145 mM sodium chloride (pH 5.5−6.5), 50 mM sodium phosphate/145 mM sodium chloride (pH 6.5−7.5), 50 mM sodium pyrophosphate/145 mM sodium chloride (pH 7.5−8.5), and 50 mM CAPS/145 mM sodium chloride (pH 9.0−10.0). The stability of the enzyme at each pH value was first determined, and subsequent determinations of k_{cat} were then only made at pH values at which the enzyme was stable over at least a 5-min period. The very low K_m values precluded full determinations of k_{cat} and K_m at each pH. Rather, rates were determined using a single, saturating substrate concentration (1 mM 2,4-DNPG) in 575 μL of buffer, reaction being initiated by the addition of enzyme (25 μL of 5 mg/mL). Values of k_{cat} were calculated after subtraction of the slow rate of spontaneous hydrolysis of 2,4-DNPG. To ensure that there was no specific buffer effect upon the reaction, rates were measured at a series of different buffer concentrations. No buffer-catalyzed reactions were observed.

Activation by Exogenous Nucleophiles. Values of k_{cat} and K_m of enzyme-catalyzed hydrolysis of a range of substrates were determined as described above, but in the presence of 200 mM sodium azide. In addition, values of K_m and k_{cat} for 2,4-DNPG were also determined in the presence of a range of sodium azide concentrations (0−200 mM). The dependence of k_{cat} on azide concentration for a range of substrates was determined at a single high substrate concentration (10 times the K_m value determined in the presence of 200 mM azide) in each case as follows: 2,4-DNPG (0.02 mM); 3,4-DNPG (0.3 mM); 2,5-DNPG (0.05 mM); 4-Cl-2NPG (0.13 mM); PNPG (0.48 mM). Further, rates of hydrolysis of 2,4-DNPG at saturating concentration (0.02 mM) were also examined by using a range of nucleophiles as follows: Meldrum's acid (0−0.07 M); dithiothreitol (0−0.198 M) with the maximum rate ($k_{cat(max)}$) observed at 0.1 M; thiophenol (0−3.75 mM), with $k_{cat(max)}$ at 3.75 mM, which is the maximum solubility of the thiophenol in buffer; β-mercaptoethanol (0−1.5 M); thiosulfate (0−2.6 M) with $k_{cat(max)}$ at 2.6 M, which is the maximum solubility of thiosulfate in buffer; sulfate (0−0.5 M) with $k_{cat(max)}$ at 0.075 M; thiocyanide (0−2.0 M) with $k_{cat(max)}$ at 1.2 M; azide (0−2.0 M) with $k_{cat(max)}$ at 0.2 M; pyridine (0−0.88 M) with

$k_{cat(max)}$ at 0.8 M; acetate (0−4.1 M) with $k_{cat(max)}$ at 1 M; benzoate (0−0.98 M) $k_{cat(max)}$ at 0.98 M; formate (0−4.3 M) with $k_{cat(max)}$ at 2.5 M; cyanide (0−0.19 M) and imidazole (0−0.66 M). Product analysis was performed by thin layer chromatography (TLC) on 60 F_{254} silica gel aluminum plates (E. Merck) run in 7:2:1 (v/v/v) ethyl acetate/methanol/water and developed with 10% H_2SO_4 in methanol. ^1H NMR spectra were obtained after removal of the enzyme from the reaction mixture by centrifugal ultrafiltration, lyophilization of the filtrate and repeated redissolution/lyophilization into D_2O.

Pre-Steady-State Kinetic Studies of E170G and E170G−Y298F Mutants. Pre-steady-state studies for both mutants were performed using the method described in the previous paper (Gebler et al., 1995) except that reaction was followed through absorption changes at 400 nm at either 5 or 37 °C. The concentration of enzyme used in each case was chosen to yield a burst with a total absorbance change of 0.06 A. The reaction was initiated by driving together 50 μL of enzyme solution and 50 μL of the appropriate concentrations of substrate in 50 mM sodium phosphate buffer, pH 7.0. The reactions were monitored by following the release of phenol product at 400 nm. Reaction rates were measured at DNPG concentrations of 0.14 × K_d − 3 × K_d for E170G, of 0.16 × K_d − 6 × K_d for E170G−Y298F. Reaction of PNPG with the double mutant was sufficiently slow to allow use of a standard Pye Unicam PU-8700 spectrophotometer for monitoring pre-steady-state kinetics. The reaction rates were determined by incubating the appropriate concentration of substrate (0.14 × K_d − 6 × K_d) in 50 mM sodium phosphate buffer, pH 7.0, at 37 °C in 1 cm microcuvettes in the thermostated block of the spectrophotometer. Reaction was initiated and then monitored at 400 nm by the addition of sufficient E170G−Y298F to provide a total absorbance change of 0.08 A. The data obtained from pre-steady-state kinetic measurements were fitted to an equation describing a first-order reaction followed by a steady state, yielding values of the pseudo-first-order rate constant (k_{obs}). Values of K_d and k_2 were determined from these k_{obs} values by direct fit to the equation $k_{obs} = k_2[S]/(K_d + [S])$ using the program GraFit (Leatherbarrow, 1990).

RESULTS

Production, Purification, and Physical Characterization of Mutant Enzymes. Following site-directed mutagenesis and transformation into *E. coli*, possible mutant colonies were picked and sequenced to confirm the presence of the desired mutation. DNA sequencing of the entire coding region of the mutant clones showed that only the desired single or double mutations were present. Western blots of small-scale cultures showed that the mutant genes were expressed at levels equivalent to that of wild-type gene. No significant differences in behavior during purification could be detected, with the proteins eluting at approximately the same position as wild-type enzyme from the ion exchange column used. Each purified protein ran as a single band on SDS−PAGE (>95% purity by inspection), at the same position (50 kDa) as native enzyme. Further, CD spectra of the two mutants (not shown) were essentially identical to that recorded for the wild-type enzyme.

The thermal stability of each mutant was estimated by monitoring changes in circular dichroism at 222 nm upon incubation at 50 °C, pH 7.0; E170G and E170G−Y298F were slightly more stable than wild-type enzyme (Figure 2).

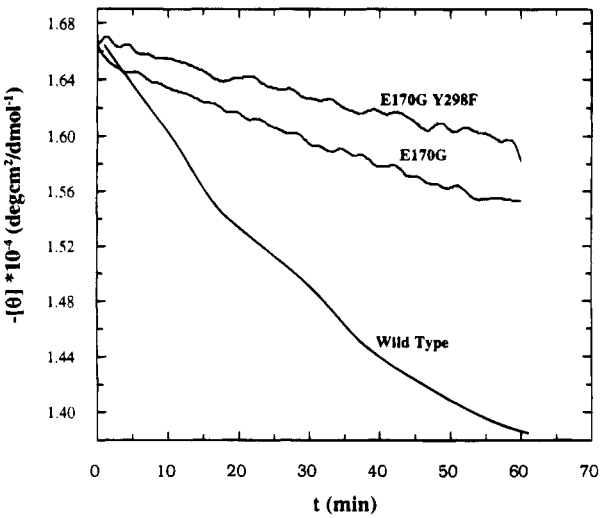

FIGURE 2: Thermal stability of native enzyme and mutants. Change in circular dichroism at 222 nm vs time at 50 °C, pH 7.0.

Active Site Titration and Stoichiometry of Incorporation of the Inactivator. Addition of 0.59 nmol of E170G to 0.58 mM DNP-2FGlu in a total volume of 250 μL resulted in the slow release of one full equivalent ($\Delta A = 0.025$, represents 0.573 nmol DNP) of dinitrophenolate according to first-order kinetics (Figure 3a), with a pseudo-first-order rate constant of $k_i = 0.091$ min^{-1}. Significant steady-state turnover could not be detected. This was consistent with a $K_i = 0.13$ mM and a $k_i = 0.19$ min^{-1}, measured independently, indicating that nitrophenolate release and inactivation were associated with the same process. Electrospray mass spectrometry gave masses of 51 122 ± 7 and 51 287 ± 8 for the mutant before and after labeling with DNP-2FGlu. The mass difference of 165 corresponds to the attachment of a single 2-deoxy-2-fluoro-D-glucopyranosyl unit (mass = 165) to the enzyme.

E170G−Y298F was analyzed in a similar manner, but with 2,4-DNPG and PNPG as titrants. Only data for PNPG are shown. One full equivalent of nitrophenolate (3.76 nmol of DNP using 3.72 nmol of the mutant, 3.85 nmol of PNP using 3.72 nmol of the mutant) was released in each case (Figure 3b). Reaction with 2,4-DNPG was sufficiently fast to require the use of stopped-flow instrumentation. Turnover in both cases was extremely slow. Electrospray mass spectrometry gave masses of 51 092 ± 8 and 51 257 ± 8 for the double mutant before and after labeling with PNPG. The mass difference of 165 corresponded to the mass (162) of one glucopyranosyl residue.

The relatively rapid release of one equivalent of dinitrophenolate by each mutant, coupled with loss of enzyme activity, showed that the activity of the mutants was intrinsic and not a consequence of contamination of an inactive mutant with a small amount of wild-type enzyme.

Substrate Reactivity. Michaelis−Menten parameters for the hydrolysis of PNPG and 2,4-DNPG by both mutants and for the hydrolysis of PG by E170G were determined wherever possible (Table 1). Values of k_{cat} for hydrolysis of 2,4-DNPG by E170G as a function of pH within the pH stability range (5.2−9.0) of the enzyme were determined (Figure 4b). No dependence upon pH is observed, in contrast to that seen with wild-type enzyme (Figure 4a)

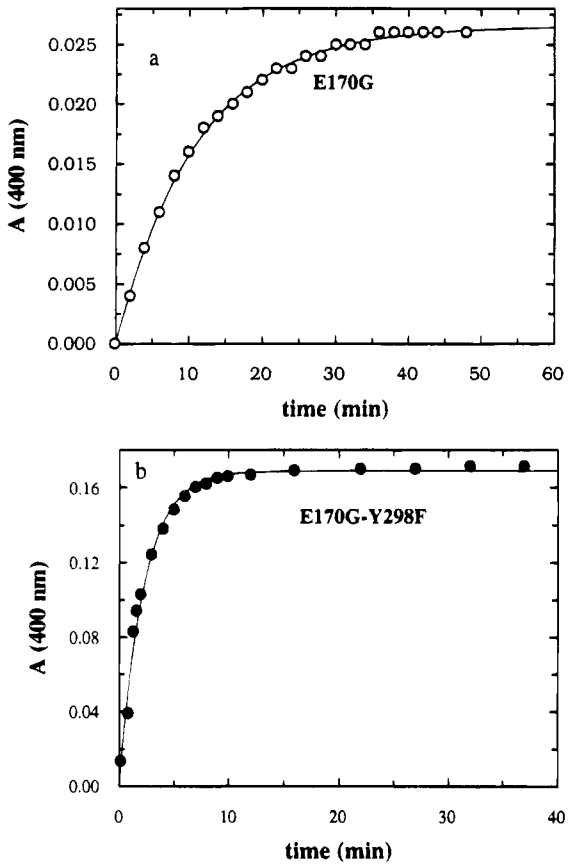

FIGURE 3: Active site titration. (a) Release of 2,4-DNP (0.57 nmol) from 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside (0.576 mM) on reaction with 0.59 nmol of E170G mutant in a total volume of 250 μL. One full equivalent corresponds to an A^{400} change of 0.026. (b) Release of PNP (3.85 nmol) from PNPG (3 mM) on reaction with 3.72 nmol of E170G−Y298F mutant in a total volume of 200 μL. One full equivalent corresponds to an A^{400} change of 0.165.

Table 1: Michaelis−Menten Parameters for the Hydrolysis of Aryl β-D-Glucopyranosides by Wild-Type *Agrobacterium* β-Glucosidase and the Mutants of E170G and E170G-Y298F

substrate	enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat}(WT)/ k_{cat}(MT[a])
PNPG	wild type[b]	159	0.078	2.04×10^3	1.0
	E170G	0.015	0.048	0.31	1.1×10^4
	Y298F[c]	0.091	0.013	7.1	1.7×10^3
	E170G Y298F	2.1×10^{-5}			
DNPG	wild type[b]	87.9	0.031	2.8×10^3	1.0
	E170G	0.05	0.0001	392	1.8×10^3
	Y298F[c]	0.030	0.001	37	2.7×10^3
	E170G Y298F	3.4×10^{-5}			2.6×10^6
PG	wild type[b]	5.4	2.12	2.6	1.0
	E170G	$<1.0 \times 10^{-6}$			$>5 \times 10^6$

[a] MT, mutant; WT, wild type. [b] Kempton and Withers (1992). [c] Gebler et al. (1995).

Pre-steady-state kinetics were also investigated and data analyzed according to the scheme shown below where E

Scheme 1

$$E + NPG \underset{}{\overset{K_d}{\rightleftharpoons}} E{\bullet}NPG \xrightarrow{k_2} E\text{-}G \xrightarrow{k_3} E + G$$
$$\downarrow$$
$$NP$$

corresponds to enzyme, NPG to nitrophenyl glucoside, NP to nitrophenol, and G to glucose (Table 2).

Effects of Added Nucleophiles on Rates of Hydrolysis. Rates of hydrolysis of 2,4-DNPG (0.2 mM = 100−1000K_m)

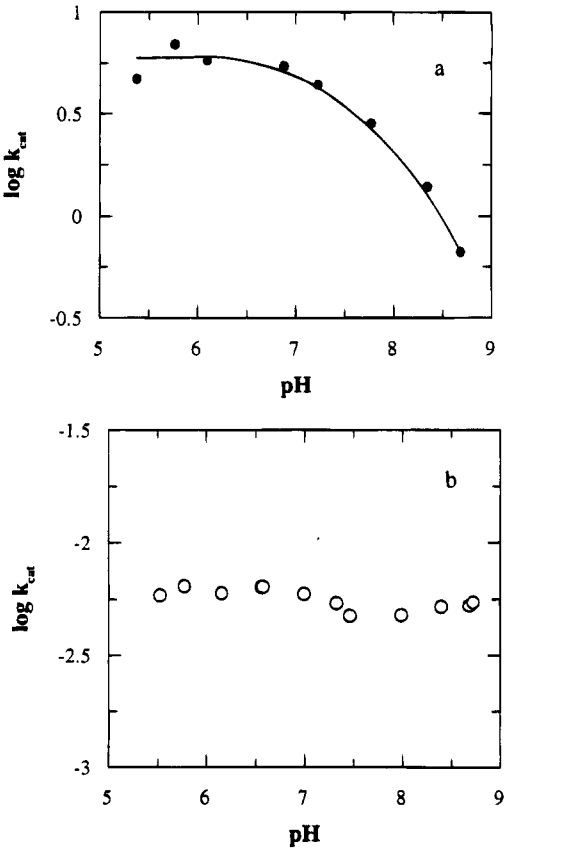

FIGURE 4: pH dependence for the hydrolysis of 2,4-DNPG. Plots of log k_{cat} vs pH for wild type (a) and E170G (b).

Table 2: Pre-Steady-State Parameters for the Hydrolysis of Aryl β-D-Glucopyranosides by Wild-Type *Agrobacterium* β-Glucosidase and the Mutants of E170G and E170G-Y298F

substrate	enzyme	k_2 (s^{-1})	K_d (mM)	k_2/K_d (s^{-1} mM^{-1})	k_2(WT)/ k_2(MT[a])
		$T = 37$ °C			
PNPG	wild type[b]	≥159		3300	1.0
	E170G[c]	≤0.015			≥1.1×10^4
	Y298F[b]	0.75	0.17	4.4	212
	E170G Y298F	8.8×10^{-3}	0.208	0.042	1.8×10^4
		$T = 5$ °C			
DNPG	wild type[b]	1300	0.65	2.0×10^3	1.0
	E170G	550	0.53	1.0×10^3	2.4
	Y298F[b]			1.1×10^3	
	E170G Y298F	86	0.05	$1.9 * 10^3$	15.1

[a] MT, mutant; WT, wild type. [b] Gebler et al. (1995). [c] No burst was observed; the value provided is the estimated k_{cat}.

by E170G were determined in the presence of a range of different nucleophiles. Large rate increases of approximately 70−300-fold were seen with carboxylate-containing nucleophiles and with azide, and more modest rate increases with most sulfur nucleophiles (Table 3). Two other potential nucleophiles tested, imidazole and cyanide, did not increase the rate and even appeared to slightly inactivate the enzyme. None of these nucleophiles affected hydrolysis by wild-type enzyme significantly.

Azide was chosen as nucleophile and 2,4-DNPG as substrate for a more detailed investigation of the rate enhancement (Figure 5). Both k_{cat} and K_m increased as a function of azide concentration, leveling off at high concentrations. In contrast, beyond a small increase at the lowest concentrations, k_{cat}/K_m remained virtually constant. The

Table 3: Apparent k_{cat} Values for the Hydrolysis of 2,4-DNPG by Glu170Gly in the Presence of Various Nucleophiles[a]

nucleophile	apparent $k_{cat(max)}$ (s^{-1})	apparent $k_{cat(max)}/k_{cat}$
no	0.05	1
Meldrum's acid	0.05	1
dithiothreitol	0.42	8
thiophenol	0.75	15
β-mercaptoethanol	0.05	1
thiosulfate	0.37	7
sulfite	0.36	7
thiocyanide	0.16	3
azide	14.9	292
pyridine	0.15	3
acetate	8.1	159
benzoate	3.4	67
formate	7.63	150

[a] Cyanide and imidazole decreased the observed rates, possibly due to inactivation of the enzyme. [b] The rates were measured at a range of concentrations of each nucleophile; the value indicated here was the observed maximum rate. The details are described under Experimental Procedures.

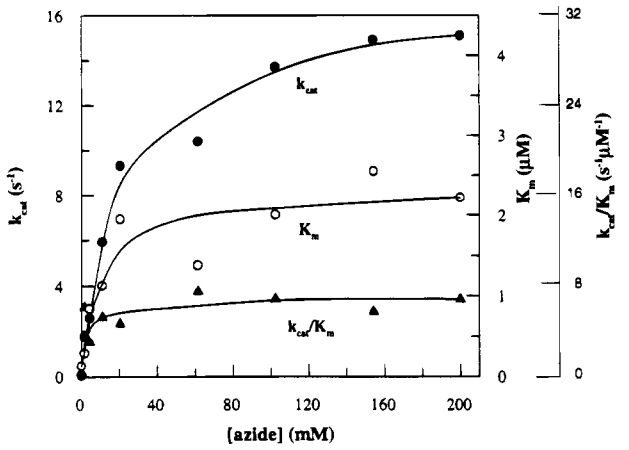

FIGURE 5: Kinetic parameters for hydrolysis of 2,4-DNPG by E170G in the presence of various concentrations of sodium azide.

Table 4: Michaelis–Menten Parameters for the Hydrolysis of Aryl β-D-Glucopyranosides by Glu170Gly Mutant in the Absence and Presence of 200 mM Azide

sodium azide	phenol substituent	pK_a	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
	2,4-dinitro	3.96	0.051	1.3×10^{-4}	392
	2,5-dinitro	5.15	0.054	1.0×10^{-4}	540
	3,4-dinitro	5.36	0.056	6.4×10^{-4}	87.5
0 mM	4-chloro-2-nitro	6.45	0.053	7.5×10^{-4}	70.6
	4-nitro	7.18	0.015	0.048	0.31
	2-nitro	7.22	0.048	0.013	3.6
	2,4-dinitro	3.96	10.8	1.7×10^{-3}	6353
	2,5-dinitro	5.15	9.89	2.7×10^{-3}	3663
	3,4-dinitro	5.36	5.95	3.0×10^{-2}	198
200 mM	4-chloro-2-nitro	6.45	6.46	1.5×10^{-2}	431
	4-nitro	7.18	0.017	0.045	0.38
	2-nitro	7.22	0.89	0.088	10.1

effects of 200 mM azide on the hydrolysis of a range of aryl β-D-glucopyranosides by E170G were also determined (Table 4 and Figure 6). Finally, k_{cat} values for hydrolysis of a range of aryl glycosides of different leaving group ability were determined at different concentrations of azide (Figure 7).

Values of k_{cat} for the hydrolysis of both 2,4-DNPG and PNPG by E170G–Y298F were determined as a function of

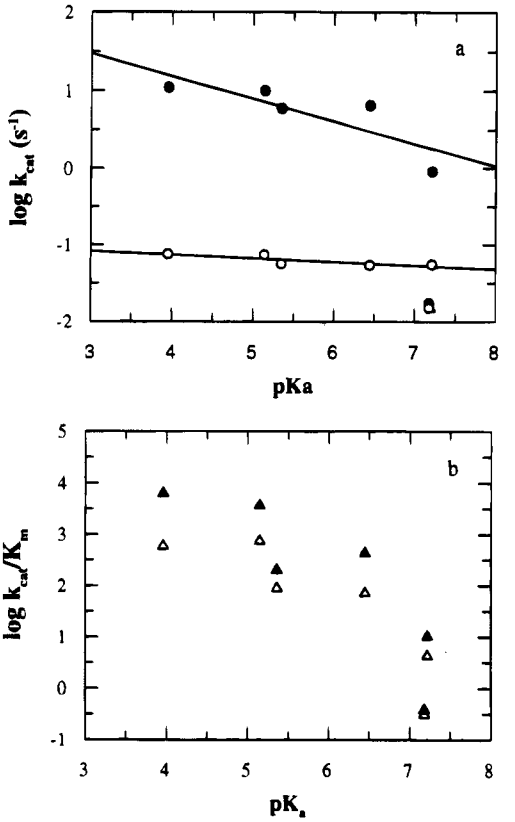

FIGURE 6: Brønsted plots relating rates of E170G-catalyzed hydrolysis of aryl glucosides with the leaving group ability of the phenol. The data are taken from Table 4. (a) Plots of log k_{cat} vs pK_a of aglycon phenol in the absence (○) and presence (●) of 200 mM sodium azide. (b) Plots of log (k_{cat}/K_m) vs pK_a of aglycon phenol in the absence (△) and presence (▲) of 200 mM sodium azide.

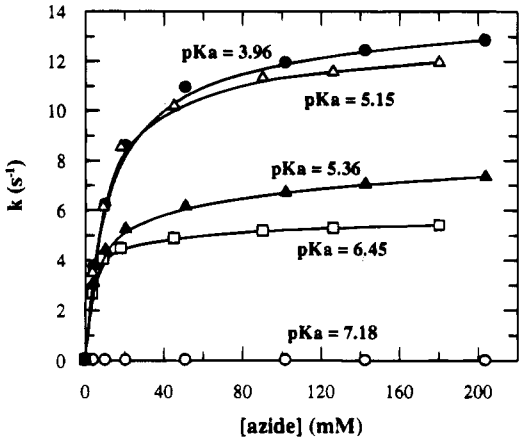

FIGURE 7: Plot of k_{cat} vs concentration of sodium azide for aryl glucosides. 2,4-DNPG (●); 2,5-DNPG (△); 3,4-DNPG (▲); 4-Cl-2NPG (□); PNPG (○).

azide concentration (Figure 8b). The rate increases were similar to those seen with E170G. The effects of azide on the glycosylation rate constant, k_2, for hydrolysis of PNPG by E170G–Y298F were analyzed by following the pre-steady-state kinetics. The very slow rate of reaction of PNPG with E170G–Y298F allowed the use of a standard spectrophotometer (Figure 8a).

Reaction Products in the Presence of Nucleophiles. Thin layer chromatographic analysis of reaction mixtures containing azide revealed the formation of a new product (R_f = 0.64), distinct from glucose (R_f = 0.15) or substrate (R_f =

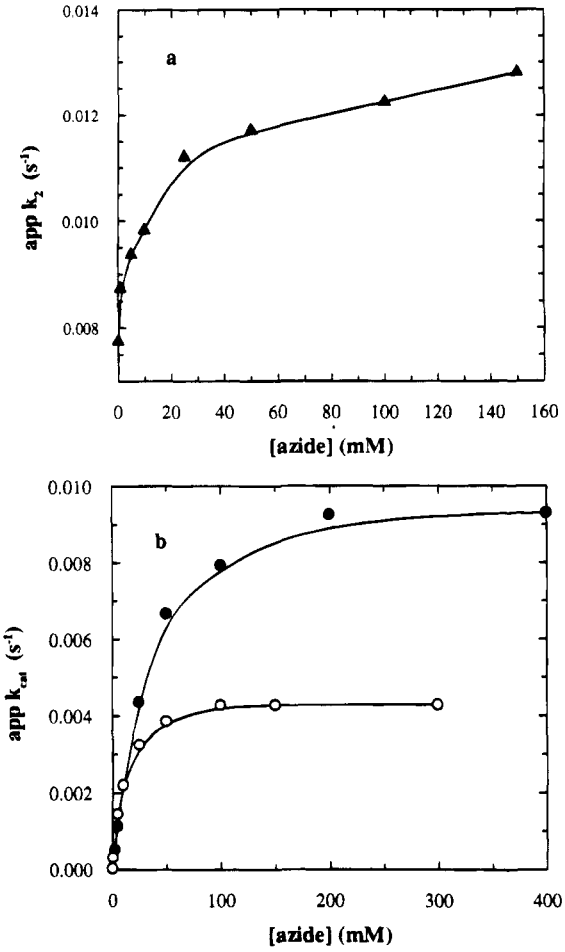

FIGURE 8: Azide effect on E170G-Y298F. (a) Plot of apparent k_2 vs concentration of azide for hydrolysis of PNPG. (b) Plot of apparent k_3 vs concentrations of azide for PNPG (○) and 2,4-DNPG (●) hydrolyzed by double mutant.

0.56) but identical to that of β-glucosyl azide obtained by conventional organic synthesis (Micheel & Klemer, 1961). The ^{1}H NMR spectrum was also consistent with the product being β-glucosyl azide: ^{1}H NMR (400 MHz, D_2O) δ: 4.64 (1 H, d, $J_{2,1}$ = 9.0 Hz, H-1), 3.89 (1 H, dd, $J_{5,6}$ = 2.2 Hz, $J_{6',6}$ = 12.4 Hz, H-6), 3.71 (1 H, dd, $J_{5,6'}$ = 5.6 Hz, $J_{6,6'}$ = 12.4 Hz, H-6'), 3.45 ~ 3.55 (2 H, m, H-3, H-5), 3.40 (1 H, t, $J_{3,4} = J_{5,4}$ = 9.2 Hz, H-4), 3.24 (1 H, t, $J_{3,2} = J_{1,2}$ = 9.0 Hz, H-2). An adduct was also detected in reaction mixtures containing thiophenol but not in those containing formate, acetate, or benzoate, possibly because of decomposition of labile acylal adducts.

DISCUSSION

The mechanism of Abg involves a two-step process (Kempton & Withers, 1992), as shown in Scheme 1 of the previous paper in this issue (Gebler et al., 1995). In the first step, a glycosyl−enzyme intermediate is formed with general acid catalytic assistance from an active site amino acid residue. In the second step, this intermediate is hydrolyzed with general base catalytic assistance, presumably provided by the same amino acid residue. As noted previously for Cex, the retaining exoglycanase from *Cellulomonas fimi* (MacLeod et al., 1994), modification of this acid/base residue will affect the rates of both steps, but the extent to which each step is affected is not necessarily equivalent. The effects of modification of this residue on the second step will necessarily be the same for all substrates of the same sugar type. However, the effects on the first step will depend upon the identity of the aglycone and its inherent leaving group ability. Substrates with poor leaving groups (alcohols of high pK_a) will be affected severely while those with good leaving groups (alcohols of low pK_a), which need little or no acid catalytic assistance, will be affected very little.

It should therefore be possible to determine whether a particular mutation has resulted in removal of the acid/base catalyst by measurement of the appropriate rate constants. This requires the identification of the rate-determining step for the substrates in question and the most direct means of assessing this is through stopped flow measurements. The pre-steady-state bursts of nitrophenolate released by E170G with 2,4-DNPG and by E170G-Y298F with both 2,4-DNPG and PNPG show that a step subsequent to dinitrophenolate release, most likely deglycosylation, is rate-limiting. The very low K_m value for hydrolysis of DNPG by E170G is also consistent with the accumulation of a glycosyl−enzyme intermediate. By contrast E170G did not release a burst of nitrophenolate with PNPG, suggesting that glycosylation is rate-limiting in that case. Further, the K_m value for hydrolysis of PNPG by E170G was very similar to that for the wild-type enzyme, as might be expected. Parallel stopped-flow studies (Namchuk and Withers, unpublished results) show that glycosylation is at least partially rate-limiting with the wild-type enzyme. The deglycosylation rate constant k_3, obtained from the k_{cat} value for 2,4-DNPG, is reduced some 1760-fold with E170G, consistent with Glu170 acting as a general base catalyst (Table 1). Effects on the glycosylation step are best estimated from relative k_2 values for each substrate (Table 2). The rate of the glycosylation step with 2,4-DNPG is reduced only 2.4-fold by removal of the carboxylate group, whereas with PNPG it is reduced by at least 10600-fold. This behavior is exactly that predicted for an acid catalyst mutant. The effect is even more striking with phenyl glucoside, a substrate with an even greater need for protonic assistance; the glycosylation step being slowed more than 5 × 10^6-fold (Table 1).

A second indicator of the removal of the acid/base catalyst would be a change in the pH dependence of the catalytic parameters for the enzyme because the pH dependence of enzymatic reactions is generally considered to reflect ionizations of acid/base groups involved in catalysis. The k_{cat} for the hydrolysis of DNPG by the wild-type enzyme is dependent on a single ionization of pK_a 8.1 in the assay pH range (Kempton & Withers, 1992), but no such dependence is seen for the mutant enzyme, suggesting that the group responsible for this pH dependence has been removed (Figure 4). Similar behavior has also been seen on mutation of acid/base catalysts in TEM-1 β-lactamase (Delaire et al., 1991) and in the recombinant protein tyrosine phosphatases from *Yersinia enterocolitica* (Zhang et al., 1994).

The third piece of evidence that Glu 170 is indeed the acid/base catalyst is the finding that k_{cat} values for hydrolysis of 2,4-DNPG by E170G increase very significantly upon addition of anionic nucleophiles (Table 3), the most effective being azide and the carboxylate nucleophiles, as seen in other enzyme systems (Huber & Chivers, 1993; MacLeod et al., 1994). No significant effects are observed with PNPG under equivalent conditions, nor are rate increases seen for hydrolysis of 2,4-DNPG by wild-type enzyme upon addition

of azide. The rate increase, coupled with the accumulation of a β-glucosyl azide product, indicates that azide and the other nucleophiles can compete effectively with water for reaction with the glycosyl−enzyme intermediate, thereby increasing the steady-state rate. Anions such as azide and carboxylates and, to a slightly lesser extent, thiolates are indeed known to react preferentially with cationic transition states (Ritchie, 1972). The fact that they do so to form products of β configuration with E170G, but not with wild-type enzyme, suggests that the replacement of Glu170 by Gly has left a cavity sufficient to accommodate small anions directly adjacent to the β-face of the glycosyl−enzyme intermediate. Such behavior would indeed be expected if Glu170 is the acid/base catalyst. Presumably, charge screening from the glutamate side chain in wild-type enzyme is sufficient to deny access to these anions. Somewhat similar effects have been seen in other systems wherein addition of exogenous reagents restores activity to mutants lacking a comparable functionality (Toney & Kirsch, 1989). However, in those cases the added species did not act as a nucleophile but as a structural moiety or an acid/base catalyst.

The plateauing of the increase in k_{cat} for the hydrolysis of aryl β-glucopyranosides by E170G at higher azide concentrations (Figures 5 and 7) cannot be due to saturation of a binding site for azide because the final rates are quite different for each substrate. Furthermore, different concentrations of azide are required for half-activation (Figure 7). It is likely that the curvature is due to a change in rate-determining step: as the concentration of azide is increased, the rate of deglycosylation increases, until glycosylation becomes rate-determining. Indeed, the final rate observed (plateau value) correlates with the leaving group ability of the aglycon, as would be expected if glycosylation becomes rate-limiting at the higher azide concentrations (Figure 7).

The lack of dependence of k_{cat} on aglycon pK_a seen in the Brønsted plots for E170G in the absence of azide suggests that deglycosylation is the rate-determining step for all substrates except PNPG (Figure 6). Azide (200 mM) increases the rates dramatically, and the increased rates now show some dependence on leaving group ability, suggesting that glycosylation is rate limiting under these conditions. The value for PNPG, however, does not increase since glycosylation is already rate-limiting in that case. The K_m value for the hydrolysis of 2,4-DNPG by E170G increases significantly as the azide concentration is increased. Such behavior is perfectly reasonable if the relative values of k_2 and k_3 are changing, since less of the glycosyl−enzyme will accumulate as k_3 increases relatively to k_2. One consequence of this is that k_{cat}/K_m values are relatively invariant with azide concentration, as revealed by the Brønsted plots (Figure 6b): the shape of the curves does not change, showing that the effect of azide is principally on the deglycosylation step because k_{cat}/K_m values reflect the first irreversible step, most likely glycosylation of the enzyme. The small increase in k_{cat}/K_m at the lowest concentration of azide suggests that azide may have a small effect on the first step. This is indicated also by the effects of azide on the hydrolysis by E170G−Y298F of PNPG, a substrate for which the glycosylation step is rate-limiting: k_{cat} and k_2 are increased 1.5- and 1.6-fold, respectively (Figure 8). Azide could slightly activate the first step in any of several ways: binding of azide in the position of the carboxylate could help stabilize a more reactive conformation or ionization state of the enzyme; alternatively, azide itself ($pK_a = 4.72$; Quintin, 1940) could provide some acid catalytic assistance once bound.

The properties of E170G clearly are consistent with Glu170 being the acid/base catalyst. It should be noted that the equivalent residue in another enzyme of family 1, Glu160 of the 6-phospho-β-galactosidase from *Staphylococcus aureus,* is thought to be the acid/base catalyst because of the 1000-fold reduction in k_{cat} and 200-fold reduction in k_{cat}/K_m for the hydrolysis of *p*-nitrophenyl 6-phospho-β-galactoside by the E160N mutant (Witt et al., 1993). Furthermore, the equivalent residue of a family 1 enzyme from Cassava was identified by tagging with the affinity label *N*-bromoacetyl β-D-glucopyranosylamine and was suggested to be the acid/base catalyst on that basis (Keresztessy et al., 1994).

It is interesting to compare the rate reductions observed upon removal of the acid/base catalyst from Abg with those found for Cex. Removal of the general base catalyst from Cex reduced the rate of the deglycosylation step, k_3, some 200-fold, whereas a 2000-fold reduction was observed for the equivalent step with Abg. Similarly, removal of the general acid catalyst reduced the rate of the glycosylation step k_2 some 20-fold for hydrolysis of PNPC by Cex but by some 10 600-fold for the hydrolysis of PNPG by Abg. With substrates having an even poorer leaving group than PNP and which really require the acid catalyst, the rate of the glycosylation step was reduced approximately 3×10^5-fold for the hydrolysis of pBrPG by Cex and approximately 5×10^6-fold for the hydrolysis of PG by Abg. Effects on DNPG and DNPC hydrolysis were similarly small in the two cases, consistent with the absence of significant protonic assistance. It seems that general acid and general base catalytic assistance are somewhat more important for Abg than for Cex, although the smaller reduction observed for Cex could be a consequence of more effective recruitment of some other active site residue as an alternative acid/base catalyst in that case. Interestingly, Brønsted plots for the two wild-type enzymes also point out significant differences, there being a much greater degree of charge development on the phenolate oxygen upon hydrolysis of aryl glucosides by Abg than upon hydrolysis of cellobiosides by Cex (relative β_{lg} values of −0.7 and −0.3, respectively). There may be a greater degree of bond cleavage at the transition state for Abg than for Cex and/or proton transfer at the transition state is more complete for Cex than for Abg.

Removal of the phenolic hydroxyl group in Y298F slowed the deglycosylation step 2000−3000-fold (relative k_{cat} values for 2,4-DNPG and PNPG with the wild-type and the mutant), but the glycosylation step was slowed only 212-fold [relative k_2 values for PNPG, Table 2 in Gebler et al. (1995)]. These changes are similar to, though slightly smaller in magnitude than, those for the Glu170Gly mutation, suggesting that Tyr298 might also play some role in acid catalysis. This could involve orientation or activation of Glu170, although the location of the residue on the α-face and the absence of an azide effect make a direct role unlikely. The effects of the two mutations on the deglycosylation step are completely additive (Table 1). Thus, combination of the 11 000-fold reduction observed for E170G relative to wild-type enzyme with the 1700-fold reduction seen for Y298F should give a 1.9×10^7-fold reduction for E170G−Y298F if the two mutations are independent. The reduction actually observed, 7.6×10^6-fold, is indeed very close to this value, suggesting

that the two residues do not interact directly and, therefore, that Tyr 298 has no role in general base catalysis. This is not true, however, for the glycosylation step, because the effects on k_2 are far from additive, a combined effect of 2.3 $\times$ 10^6 being predicted but an effect of 1.8 $\times$ 10^4 actually being observed (Table 2). Thus, once the true acid catalyst (Glu170) is changed, changing Tyr298 has no additional effect. Whatever the correct interpretation, the glycosyl–enzyme formed with E170G–Y298F is extremely stable, with a half-life for hydrolysis of 7.1 h, thus opening the way to investigating the structure and properties of the glucosyl–enzyme intermediate species using a simple glucoside. Such studies are ongoing.

Interestingly, after these studies were completed, the sequences of several new family 1 glycosidases became available, including two in which the residue corresponding to Glu170 is replaced by glutamine (see Figure 1) (Xue et al., 1992; Falk et al., 1992). These two enzymes are both thioglucosidases hydrolyzing glucosinolates, substrates in which an anionic aglycon is attached to the sugar via a sulfur linkage, such as that shown in Scheme 1. Results from this laboratory (Wang & Withers, 1995) indicate that substrates with negatively charged aglycons bind very poorly to glycosidases, possibly because of charge repulsion from the two active site carboxylates. The mutation to the amide therefore provides a means of removing the repulsive interaction and allowing the substrate to bind. The required acid catalysis might then be provided by the substrate itself in an example of Nature's own "substrate-assisted catalysis".

ACKNOWLEDGMENT

We thank K. Rupitz for technical assistance. We also thank the Natural Sciences and Engineering Research Council of Canada and the Protein Engineering Network of Centres of Excellence for financial support.

REFERENCES

Baird, S. D., Hefford, M. A., Johnson, D. A., Sung, W. L., Yaguchi, M., & Seligy, V. L. (1990) *Biochem. Biophys. Res. Commun. 169*, 1035.

Day, A. G., & Withers, S. G. (1986) *Can. J. Biochem. Cell Biol. 64*, 914.

Delaire, M., Lenfant, F., Labia, R., & Masson, K. (1991) *Protein Eng. 4*, 805.

Falk, A., Xue, J., Lenman, M., & Rask, L. (1992) *Plant Sci. 83*, 387.

Gebler, J. C. Trimbur, D. E., Warren, R. A. J., Aebersold, R., Namchuk, M., & Withers, S. G. (1995), *Biochemistry 34*, 14547–14553.

Graham, R., Greenwood, J. M., Warren, R. A. J., Kilburn, D. G., & Trimbur, D. E. (1995) *Gene, 158*, 51.

Henrissat, B. (1991) *Biochem. J., 280*, 309.

Henrissat, B., & Bairoch, A. (1993) *Biochem. J. 293*, 781.

Huber, R., & Chivers, P. (1993) *Carbohydr. Res. 250*, 9.

Kempton, J. B., & Withers, S. G. (1992) *Biochemistry 31*, 9961.

Keresztessy, Z., Kiss, L., & Hughes, M. A. (1994) *Arch. Biochem. Biophys. 315*, 323.

Kunst, A., Draeger, B., & Ziegenhorn, J. (1984) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) 3rd ed., Vol. 6, pp 163, Weinheim, Deerfield Beach, FL.

Laemmli, K. (1970) *Nature 277*, 680.

Leatherbarrow, R. J. (1990) Grafit Version 2.0, Erithacus Software Ltd., Staines, U.K.

Legler, G. (1990) *Adv. Carb. Chem. Biochem. 48*, 319.

Lehtovaara, P. M., Koivala, A. K., Baford, J., & Knowles, J. K. C. (1988) *Protein Eng. 2*, 63.

MacLeod, A. M., Lindhorst, T., Withers, S. G., & Warren, R. A. J. (1994) *Biochemistry 33*, 6571.

Micheel, F., & Klemer, A. (1961) *Adv. Carbohydr. Chem. 16*, 85.

Quintin, M. (1940) *C. R. Acad. Sci. Ser. D210*, 625.

Ritchie, C. D. (1972) *Acc. Chem. Res. 5*, 348.

Sinnott, M. L. (1990) *Chem. Rev. 90*, 1171.

Street, I. P., Kempton, J. B., & Withers, S. G. (1992) *Biochemistry 31*, 9970.

Toney, M. D., & Kirsch, J. F. (1989) *Science 243*, 1485.

Trimbur, D., Warren, R. A. J., & Withers, S. G. (1992) *J. Biol. Chem 267*, 10248.

Wang, Q., & Withers, S. G. (1995) *J. Am. Chem. Soc.* (in press).

White, A., Withers, S., Gilkes, N., & Rose, D. (1994) *Biochemistry 33*, 12546.

Withers, S. G., & Street, I. P. (1988) *J. Am. Chem. Soc. 110*, 8551.

Withers, S. G., Warren, R. A. J., Street, I. P., Rupitz, K., Kempton, J. B., & Aebersold, R. (1990) *J. Am. Chem. Soc. 112*, 5887.

Witt, E., Frank, R., & Hengstenberg, W. (1993) *Protein Eng. 6*, 913.

Xue, J., Lenman, M., Falk, A., & Rask, L. (1992) *Plant Mol. Biol. 18*, 387.

Zhang, Z.-Y., Wang, Y., & Dixon, J. E. (1994) *Proc. Natl. Acad. Sci. U.S.A., 91*, 1624.

BI951172P