Substrate-Induced Inactivation of a Crippled β-Glucosidase Mutant: Identification of the Labeled Amino Acid and Mutagenic Analysis of Its Role†

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ABSTRACT: The β-glucosidase from *Agrobacterium sp.* catalyzes the hydrolysis of β-glucosides via a covalent α-D-glucopyranosyl−enzyme intermediate involving Glu358. Hydrolysis of 2,4-dinitrophenyl β-D-glucopyranoside by the low activity Glu358Asp mutant of *Agrobacterium* β-glucosidase is accompanied by time-dependent inactivation of the enzyme. Through kinetic studies, labeling, and sequence analysis, inactivation is shown to be a consequence of the occasional (1 time in 1100) attack of Tyr298 on the anomeric center of the substrate, in place of the catalytic nucleophile, with formation of a stable α-D-glucopyranosyl tyrosine residue. Tyr298 is conserved throughout family 1 of glycoside hydrolases, an indication of a possible role in catalysis. Results of a kinetic analysis of the Tyr298Phe mutant are consistent with a function of Tyr298 in both orienting the nearby nucleophile Glu358 and stabilizing its deprotonated state in the free enzyme.

Agrobacterium faecalis β-glucosidase (Abg)[1] is a "retaining" glycosidase which catalyzes the hydrolysis of β-glucosidic linkages with overall retention of anomeric configuration (Withers et al., 1986). The accepted mechanism of action involves a double displacement reaction in which the enzyme forms and hydrolyzes a glycosyl−enzyme intermediate via oxocarbenium ion-like transition states (Scheme 1) (Withers et al., 1986; Sinnott, 1990; Kempton & Withers, 1992; McCarter & Withers, 1994). Direct evidence for the formation of such a covalent α-D-glycopyranosyl−enzyme intermediate has been obtained by ^{19}F NMR study of the catalytically competent intermediate which accumulates during hydrolysis of 2,4-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside (Withers & Street, 1988), and the nucleophilic residue involved has been identified as Glu358 by sequence analysis of the labeled peptide (Withers et al., 1990). No three-dimensional structural information is available on this β-glucosidase; thus, information on active site residues has come from labeling studies, from sequence alignments within the family of related amino acid sequences (Henrissat & Bairoch, 1993), and, more recently, from site-directed substitution of Glu358 by Asn, Gln, and Asp (Withers et al., 1992), and from random point mutations in the region flanking this residue (Trimbur et al., 1992). The amide mutants at position 358 are essentially inactive, confirming the importance of this residue. However, the Asp mutant is still active, but at a considerably reduced level. Detailed kinetic analysis of this mutant (Withers et al., 1992) revealed that the rate constant for the first step, formation of the glycosyl−enzyme, is approximately 2500 times lower than that of the native enzyme, again indicating the importance of the nucleophile and of its precise placement. Interestingly, although DNPGlu was the most active substrate with Glu358Asp, it also slowly inactivated the enzyme in a time-dependent manner, while the mutant incubated in the absence of substrate was quite stable. Fortunately, this inactivation was slow enough not to affect the initial rate kinetics significantly, allowing detailed kinetic evaluation. No such inactivation behavior was seen with less activated substrates, such as PNPGlu, or with glucose or 2,4-dinitrophenol.

These preliminary results suggested that inactivation might be a consequence of occasional derivatization of an alternative nucleophilic group in the active site of this mutant in which the true nucleophile at position 358 is mispositioned. Identification of the alternative nucleophile would therefore provide further insight into residues present at the active site of the enzyme. This paper describes the investigation of the mode of substrate-induced inactivation, the identification of the amino acid residue involved, and subsequent construction and analysis of mutants modified at that position.

MATERIALS AND METHODS

o-Nitrophenyl, *p*-nitrophenyl, and phenyl glucosides, gluconolactone, X-Glu, and α-glucosidase (EC 3.2.1.20, type

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[1] Abbreviations: X-Glu, 5-bromo-4-chloro-3-indolyl β-D-glucopyranoside; IPTG, isopropyl β-D-thiogalactopyranoside; DNP, 2,4-dinitrophenol; DNPGlu, 2′,4′-dinitrophenyl β-D-glucopyranoside; PNPGlu, 4′-nitrophenyl β-D-glucopyranoside; [^{3}H]DNPGlu, [1-^{3}H]2′,4′-dinitrophenyl β-D-glucopyranoside; [^{2}H]DNPGlu, [1-^{2}H]2′,4′-dinitrophenyl β-D-glucopyranoside; Glu358Asp, Glu358Asp mutant of β-glucosidase from *Agrobacterium faecalis*; Tyr298Phe, Tyr298Phe mutant of β-glucosidase from *Agrobacterium faecalis*; Abg, *Agrobacterium faecalis* β-glucosidase; TYP, tryptone, yeast extract, K_2HPO_4; ESIMS, electrospray ionization mass spectrometry.

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III from bakers yeast) were obtained from Sigma Chemical Co. Buffer chemicals were obtained from BDH and were used without further purification. Growth media components were obtained from Difco. Restriction enzymes, polymerases, and nucleotides were from BRL and Pharmacia. Radionucleotides were from New England Nuclear Corp. DNPGlu was synthesized as previously described (Kempton & Withers, 1992); [^{3}H]DNPGlu, in which the tritium label is incorporated at the anomeric center of the sugar, was synthesized using the literature procedure for the preparation of [^{2}H]DNPGlu, but where sodium borotritiide (Amersham Life Sciences) was substituted for sodium borodeuteride (Berven & Withers, 1986). All other aryl glucosides were obtained using previously published protocols (Kempton & Withers, 1992). Concentrations of Abg and its mutants were estimated by absorbance at 280 nm using an extinction coefficient of $\epsilon = 2.2$ cm^2 mg^{-1} (Withers et al., 1987). The Glu358Asp mutant of Abg was expressed in *Escherichia coli* and isolated and assayed for activity as previously described (Withers et al., 1992). Stopped-flow measurements were carried out using an Applied Photophysics MV 17 microvolume stopped-flow spectrophotometer equipped with a constant temperature water bath. Radioactivity (^{3}H) was determined by liquid scintillation counting. Peptide sequences were determined by gas−liquid phase Edman degradation on an Applied Biosystems model 477A sequenator using standard protocols with on-line PTH detection using an Applied Biosystems Model 120A analyzer.

Inactivation of Glu358Asp. Time-dependent inactivation of Glu358Asp by high concentrations of DNPGlu was monitored by incubating the enzyme (0.044 nmol) in 50 mM sodium phosphate, pH 7.0, at 37 °C in the presence of 1 mM DNPGlu. Residual enzyme activity at different times was determined by removal of a 10 μL aliquot of this mixture, addition to a solution of DNPGlu (0.2 mM, 700 μL) in the same buffer in a 1-cm cell, and monitoring the release of DNP by the change in absorbance at 400 nm. Simultaneously, the concentration of DNP in the inactivation mixture was determined by measuring the optical density at 400 nm of 10 μL aliquots diluted to 500 μL in buffer.

Identification of Attachment Site. Glu358Asp (500 μg) was reacted with 580 μL of [^{3}H]DNPGlu (17 mM, specific activity 11 mCi/mmol; 1000-fold molar excess) in sodium phosphate buffer, pH 7 (700 μL total volume) for 60 min, by which time the enzyme was 75% inactivated (a control sample of enzyme in buffer alone retained full activity over this time period). The enzyme solution was then dialyzed against 1 L of sodium phosphate buffer (50 mM, pH 2.1) overnight with one change. The resulting protein solution was treated with pepsin (5 μg) for 2 h at 25 °C. The peptide mixture generated was separated by HPLC using a Vydac C_4 column (5 μm, 300 Å, 4.6 × 250 mm) and eluted with a gradient of 0.1% trifluoroacetic acid in water to 0.08% trifluoroacetic acid in 70% acetonitrile/30% water. One radioactive peptide-containing fraction was obtained, and this fraction was further purified by rechromatography on a microbore HPLC system equipped with a Reliasil C_{18} column (5 μm, 300 Å, 1 × 50 mm) using the previously described solvent system. This provided a single radiolabeled peptide of suitable purity for sequencing.

Stoichiometry of Incorporation of Inactivator. Glu358Asp (1 mg, 0.02 mmol) was treated with 1.6 mL of 25 mM DNPGlu in sodium phosphate buffer, pH 7 (final volume 1.9 mL) for 12 h at 37 °C, by which time the enzyme was >99% inactivated. Excess inactivator was removed from the labeled enzyme by overnight dialysis in sodium phosphate buffer (50 mM, pH 7) with one change of buffer (final enzyme concentration was 0.4 mg/mL). A sample (20 μL) was injected into an ion spray mass spectrometer (Sciex API III) via a microbore HPLC system equipped with a polystyrenedivinylbenzene reversed-phase column (5 μm, 300 Å, 1 × 50 mm) using the TFA/water/acetonitrile gradient discussed earlier. Nonmodified Glu358Asp and native β-glucosidase samples were also analyzed in an identical manner.

Strains and Culture Conditions. E. coli strains JM101 (Viera & Messing, 1987) and RZ1032 (Kunkel, 1987) have been described. *E. coli* R1360 (araD, lac-proD, bgl-1, thi, strA) was obtained from Dr. Bodo Rak (Institut für Biologie III, Albert-Ludwigs-Universität, Schänzlestrasse 1, D-7800 Freiburg i. Br., Germany). Plasmid pTZ18R::Abg was constructed by inserting the coding sequence of the *abg* gene from pABG5 (Wakarchuk et al., 1986) into pTZ18R (Mead et al., 1986). JM101 was maintained on M9 minimal medium (Viera & Messing 1987). Plasmid containing strains were grown in Luria broth (Miller, 1972) containing 100 μg of ampillicin/mL.

DNA Techniques and in Vitro Mutagenesis. Single-stranded DNA was isolated by the following method. Cultures were grown 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 ampicillin and 10^9 PFU/mL helper phage M13K07 (Viera & Messing, 1987). Kanamycin (50 μg/mL) was added 1 h after inoculation, and the culture was grown 6−10 h at 37 °C. Phagemid were precipitated with 1.7 M ammonium acetate and 12% (w/v) PEG-6000. Single-stranded DNA was isolated from the phagemid by the method of Kristensen et al. (1987). Uracil-containing template was generated by growing the plasmid in strain RZ1032(*dut*$^-$ *ung*$^-$). Site-directed mutants were generated by the method of Kunkel (1987) with modifications for phagemid vectors (McClary et al., 1989). Specific mutations of Tyr-298 were carried out with a degenerate oligonucleotide primer: pGCCTGAATTATTNCACGCCGATGCG (N indicates the position where all four nucleotides were used). T7 DNA polymerase was used instead of the Klenow fragment of DNA Pol I for the extension reactions. After *in vitro* mutagenesis, the plasmid DNA was transformed into JM101. Transformants were selected on LB agar containing 2% X-Glc, 1 mM IPTG, and 100 μg of ampillicin/mL. Possible mutants were screened by single-track sequencing and confirmed by complete sequencing reactions. The 700 bp *StuI*−*EcoRI* fragment of *abg* was sequenced to confirm that only the desired mutation was present and then was subcloned into the expression vector pTug10Nabg. DNA was sequenced by the procedure of Tabor and Richardson (1987) with the following modifications: the reaction temperature was increased to 43 °C and deaza-7-dGTP replaced dGTP. Expression levels of mutant proteins were monitored by SDS−PAGE (Laemmli, 1970) followed by Western blot analysis (Gilkes et al., 1988) with wild-type Abg as a control.

Production and Purification of Abg. Wild-type and mutant proteins were expressed in *E. coli* R1360 from the *tac* promoter of the expression vector pTug (Trimbur et al., unpublished results). Cells were grown overnight in 2 mL of TYP-ampillicin at 30 °C. One milliliter of a culture was

used to inoculate 1 L of TYP-ampillicin. Cultures were grown for 6 h, transferred to a 15 L fermentor (Chemmap) containing 10 L of the same medium, and grown to an OD_{600} of 5. IPTG (0.1 mM) was added and the fermentation was continued for another 5 h. Cells were harvested with a Sharples continuous centrifuge at 31000*g*; the cell paste was stored at −20 °C. The enzymes were purified essentially according to the protocol used previously for wild-type Abg (Kempton & Withers, 1992), except that a larger DEAE column (5 × 30 cm) was used because of the higher level of protein produced with pTug. Enzymatic activity during purification was determined by assaying with DNPGlu.

Thermal Stability. The thermal stabilities of the native enzyme, Glu358Asp, and Tyr298Phe were determined by incubation of the enzymes at the defined temperature, removing aliquots at time intervals, and assaying these for residual activity at 37 °C in the usual manner. Thermal stability was also monitored using a circular dichroism spectrometer (JASCO) by monitoring changes at 222 nm on incubation at 50 °C in sodium phosphate buffer pH 7.

Kinetic Studies of Tyr298Phe. All kinetic studies were performed by following changes in UV/VIS absorbance with a Pye-Unicam PU-8800 spectrophotometer, equipped with a circulating water bath which maintained the cells of 1 cm path length at 37 °C. The buffer employed for all kinetic experiments was 50 mM sodium phosphate, pH 6.8. Extinction coefficients for phenols and glycosides were reported earlier (Kempton & Withers, 1992). All data were evaluated by nonlinear regression analysis using the programme GraFit (Leatherbarrow, 1990). Kinetic parameters for substrate hydrolysis and for mechanism-based inactivation were determined using the previously reported methods (Kempton & Withers, 1992). The secondary deuterium kinetic isotope effect was determined by measuring the rates of hydrolysis of 2,4-DNPGlu (1 mM, 750 μL, alternately protiated and deuterated) after the addition of enzyme (34 μg). Measurements were made seven times each for protio and deutero substrates, and the reported isotope effect represents the average of these values.

pH Stability and Activity of Tyr298Phe. Tyr298Phe was incubated at a series of pH values, and aliquots were removed at time intervals and then assayed under standard conditions. Buffers employed were as follows: pH 5.5−6.0, 100 mM MES; 6.5−8.5, 100 mM sodium phosphate; 9.0−10.0, 100 mM sodium bicarbonate. The effects of pH on catalytic activity were evaluated by assaying activity at various pH values using DNPGlu as substrate. Rates were calculated using the extinction coefficients reported previously (Tull & Withers, 1994). The enzyme was preincubated at 37 °C for 15 min at the new pH before each activity measurement.

Pre-Steady-State Kinetics. All measurements were made in 50 mM sodium phosphate buffer at either 5 or 37 °C, with a stop volume of 100 μL. The reaction was followed by observing the change in absorbance at 360 nm. Enzyme concentrations were chosen to give a burst with a total absorbance change of at least $\Delta A = 0.035$. Rates were determined using five or more substrate concentrations; the reported values are the mean of four determinations. Observed kinetic parameters (K_d, k_2) were calculated by direct fit to the appropriate expression using the program GraFit (Leatherbarrow, 1990).

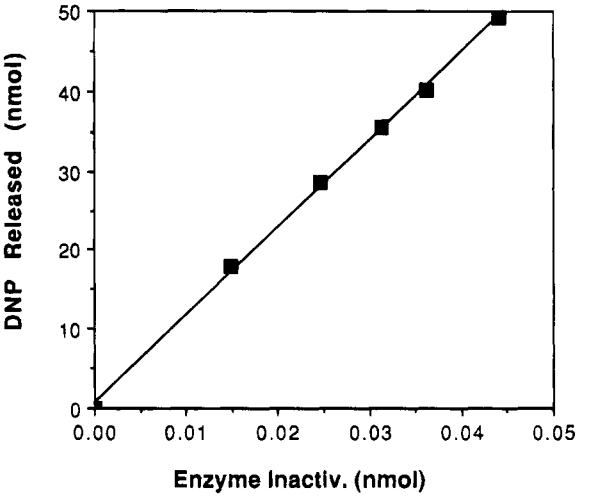

FIGURE 1: Determination of the "kill ratio" for reaction of DNPGlu with Glu358Asp. A plot of dinitrophenolate released as a function of enzyme inactivated.

RESULTS

Inactivation of Glu358Asp. Incubation of Glu358Asp with a large molar excess of DNPGlu (2000×) resulted in both hydrolysis of the substrate and time-dependent inactivation of the enzyme. A plot of the extent of the inactivation reaction (nmol of inactivated enzyme produced) versus the extent of turnover of DNPGlu (nmol of DNP released) was linear (Figure 1). The slope of this plot yielded a "kill ratio" of 1100:1, showing that one inactivation event took place for every 1100 turnovers. The mass of the enzyme after complete inactivation with DNPGlu was 51 347 ± 3, which was 163 Da greater than that of the untreated Glu358Asp mutant (51 184 ± 4), showing the stoichiometry of inactivation to be one. Inactivated Tyr298Phe did not recover activity after dialysis to remove excess inactivator and incubation in buffer, an indication of the irreversible, covalent nature of the inactivation process. Nor was reactivation

Scheme 1

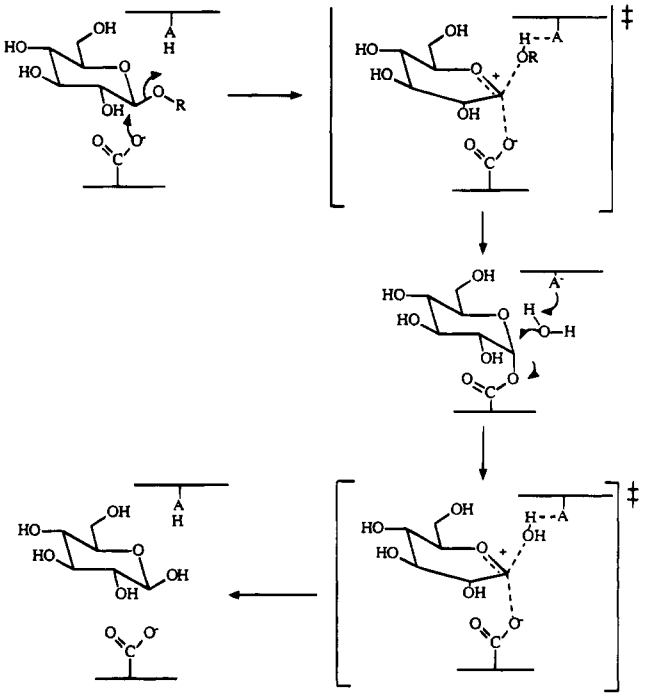

Table 1: Sequencing Data

cycle	PTH derivative	yield (pmol)	DNA sequence[a]
1	Tyr	217	Tyr
2	X[b]	100	Tyr
3	Thr	Q[c]	Thr
4	Pro	208	Pro
5	Met	208	Met
6	Arg	Q[c]	Arg
7	Val	180	Val
8	Ala	185	Ala
9	Asp	100	Asp
10	Asp	106	Asp
11	Ala	87	Ala
12	Thr	Q[c]	Thr
13	Pro	35	Pro
14	Gly	36	Gly
15	Val	23	Val
16	Glu	21	Glu
17	Phe	13	Phe

[a] Sequence of peptide derived from the DNA starting at residue 297 (Wakarchuk *et al*, 1986). [b] X = Unidentified residue. [c] Qualitative determination.

facilitated by incubation with glucosyl derivatives which do facilitate recovery of activity in DNP2F Glu-inactivated Abg via a transglycosylation process (Street et al., 1992).

Identification of Attachment Site. Labeling of a moderate quantity (500 μg) of Glu358Asp with an 860-fold excess (mol/mol) of [^{3}H]DNPGlu for 2 h resulted in ≈75% inactivation. The N-terminal amino acid sequence of the single radioactive peptic peptide obtained from the inactivated enzyme (see Materials and Methods) was Y-X-T-P-M-R-V-A-D-D-A-T-P-G-V-E-F. A nonstandard polar PTH derivative eluted in the second cycle of Edman degradation (Table 1), accompanied by a "burst" of radioactivity. The sequence aligns perfectly with residues 297−313 of Abg, where residue 298 is a tyrosine (Wakarchuk et al., 1986).

Stereochemistry of Attachment. The radioactive peptic peptide obtained from the [^{3}H]DNPGlu-labeled Glu358Asp had a mass of 1359.0 ± 0.4, consistent with expectations for the amino acid sequence determined, plus a glucose residue. Treatment of the peptide with yeast α-glucosidase reduced the mass to 1196.0 ± 0.4, consistent with removal of the glucosyl moiety, whereas treatment of the peptide with wild-type β-glucosidase resulted in no change in the mass of the peptide. This indicates an α-glucosidic linkage in the conjugate.

Mutagenesis and Mutant Isolation. Site-directed mutagenesis was used to change Tyr298 to Phe298 in wild-type Abg, as described in Materials and Methods. Possible mutants were first screened on X-Glu plates, and white colonies, indicating loss of activity, were picked and sequenced to confirm the presence of the desired mutation. All white colonies screened contained the correct mutation, and sequencing of the entire coding region of the mutant *abg* clones showed that only the single mutation was present. Western blots of small scale cultures showed that the mutant protein was expressed at levels equivalent to wild-type protein. The behaviors of the mutant and wild-type enzymes during purification were virtually identical. The purified mutant protein ran as a single band on SDS−PAGE (>95% purity by inspection), at the same position (50 kDa) as native enzyme. The CD spectrum of the mutant was essentially indistinguishable from that of the native enzyme. The thermal stability of Tyr298Phe, monitored by changes in circular dichroism at 222 nm upon incubation at 50 °C, was similar to that of the wild-type enzyme. The mutant also showed similar pH stability to that of the wild-type enzyme, retaining full activity for 30 min at 37 °C from pH 5.5 to 9.0, but dropping sharply above pH 9.5.

Substrate Specificity, Reactivity, and Inactivation Studies. Kinetic parameters for hydrolysis by Tyr298Phe of a range of substrates with different glycone moieties are presented in Table 2 and are compared with those of wild-type Abg (Kempton & Withers, 1992). Similarly, kinetic parameters for the hydrolysis by Tyr298Phe of a series of aryl glucosides of differing leaving group ability are presented in Table 3 along with equivalent values for wild-type enzyme. Although rate constants for the mutant are much smaller than those for wild-type Abg, the activities of both enzymes on these substrates (k_{cat}/K_m values) are clearly strongly dependent upon the leaving group abilities of the substrate aglycons. Pre-steady-state kinetic analysis of the hydrolysis of PNPGlu by Tyr298Phe according to Scheme 2 below gave kinetic

Scheme 2

$$E + PNPGlu \underset{}{\overset{K_d}{\rightleftharpoons}} E{\bullet}PNPGlu \xrightarrow{k_2} E\text{-}Glu \xrightarrow{k_3} E + Glu$$
$$\downarrow$$
$$PNP$$

constants of $K_d = 0.17$ mM and $k_2 = 0.75$ s^{-1}.

Equivalent individual kinetic parameters could not be determined for the wild-type enzyme because hydrolysis was too fast, but a k_2/K_d value of 3300 mM^{-1}s^{-1} was obtained.

The kinetic parameters for the inactivation of Tyr298Phe by DNP2FGlu were $k_i = 0.021$ s^{-1} and $K_i = 1.9$ mM, determined according to Scheme 3 below. The equivalent

Scheme 3

$$E + I \overset{K_i}{\rightleftharpoons} E{\bullet}I \xrightarrow{k_i} E\text{-}I$$

parameters for the wild type enzyme were $k_i = 0.42$ s^{-1} and $K_i = 0.05$ mM.

The pH dependence of the enzyme was determined by measuring rates at a saturating (1 mM) concentration of DNPGlu at each of a number of pH values, thereby yielding the plot of k_{cat} vs pH shown in Figure 2. Data were fitted to an expression relating activity to two pK_a values by nonlinear regression analysis using the program GraFit (Leatherbarrow, 1990). Values of p$K_1 = 6.2$ and p$K_2 = 8.8$ were obtained. Full determination of k_{cat} and K_m values at each pH was not feasible due to the extreme difficulties associated with such measurement at low K_m values. The secondary deuterium kinetic isotope effect determined for hydrolysis of DNPGlu by Tyr298Phe was found to be $k_H/k_D = 1.03 \pm 0.01$. This compares with a value for the wild-type enzyme of $k_H/k_D = 1.10 \pm 0.01$ Kempton & Withers, 1992).

DISCUSSION

Two likely mechanisms are possible for the inactivation of Glu358Asp in the presence of DNPGlu. One would involve attack of a nucleophilic residue at the anomeric center of the sugar, with release of the dinitrophenolate and formation of a glycosylated enzyme. The other would involve attack of a nucleophile at the C-1′ position of the dinitrophenyl moiety in a nucleophilic aromatic substitution reaction, with expulsion of the sugar and formation of a dinitrophenylated protein. The mass increase of 163 observed is entirely consistent with the attachment of a glucosyl residue (162) but could also be consistent with the attachment

Table 2: Michaelis–Menten Parameters for 4-Nitrophenyl Glycosides with Tyr298Phe β-Glucosidase

	wild type			Tyr298Phe		
substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
PNPXyl	1.52	0.24	6.4	0.0014	0.03	0.047
PNPFuc	110	0.12	905	0.22	0.014	16.2
PNPGal	215	5.0	43	1.2	8.3	0.15
2dPNPGlu	0.025	0.016	1.56	0.00026	0.07	0.0037

Table 3: Michaelis–Menten Parameters for Aryl Glucosides with Tyr298Phe β-Glucosidase

phenol	phenol	wild type			Tyr298Phe		
substituent	pK_a	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
2,4-dinitrophenyl	3.96	88	0.031	2800	0.033	0.001	37
4-nitrophenyl	7.18	169	0.078	2200	0.091	0.013	7.1
2-nitrophenyl	7.22	111	0.033	3400	0.041	0.012	3.4
3-nitrophenyl	8.39	108	0.19	571	0.053	0.024	2.2
4-chlorophenyl	9.38	29	0.56	52	0.013	0.28	0.046
phenyl	9.99	5.4	2.1	2.6	0.015	1.3	0.012

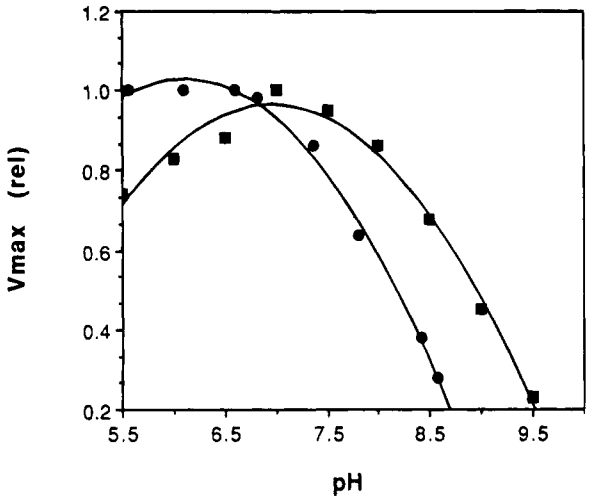

FIGURE 2: pH Dependence of V_{max} for DNPGlu hydrolysis by Abg and its Tyr298Phe mutant: (●) = Abg; (■) = Tyr298Phe mutant.

of the dinitrophenyl group (167), given the error limits of this measurement. An unambiguous answer was provided by derivatisation of the enzyme with [^{3}H]DNPGlu (containing the radiolabel in the sugar). After inactivation and dialysis, the radiolabel was found to be associated with the protein. These results, in conjunction with the mass spectrometric analysis, therefore show that inactivation is a consequence of the attachment of a single glucose residue to the protein.

The amino acid derivatized in this manner, Tyr298, is conserved throughout family 1 of glycoside hydrolases within a highly conserved region (Henrissat & Bairoch, 1993). There are two possible routes by which the tyrosine hydroxyl group could react with the sugar. In one, the hydroxyl group of Tyr298, rather than the mispositioned catalytic nucleophile Asp 358, occasionally attacks DNPGlu bound in the active site, forming a stable α-D-glucopyranosyl–enzyme intermediate on the tyrosine, thereby inactivating the enzyme. In the second, the attack occurs at a later stage, after the mutant enzyme forms a "normal" glycosyl–enzyme intermediate with Asp 358; differences in the environment of this intermediate from that in the wild-type enzyme could allow attack not only by water, but also, 1 in 1100 times, by the tyrosine hydroxyl group, resulting in the formation of an inactive, stable β-D-glucopyranosyl–enzyme intermediate. The second route would seem to be unlikely, given that Glu358Asp is inactivated by the most activated substrate tested, 2,4-DNPGlu, but not by PNPGlu. If the inactivation were a consequence of partitioning of the glycosyl–enzyme intermediate between attack by water and by Tyr298, then Glu358Asp should be inactivated by both of these substrates. Futhermore, formation of an α-D-glucosidic linkage between Tyr298 and the sugar requires direct attack on the substrate and not on the glycosyl–enzyme intermediate.

It is clear that the inactivation results from occasional misglycosylation of an active site tyrosine in the absence of a correctly positioned catalytic nucleophile, and that the tyrosine is located below the bottom (α-) face of the bound sugar, in close proximity to Asp358. Indeed, Asp358 may well provide some inadvertent general base catalytic assistance to the attack of tyrosine. In the wild-type enzyme, however, Tyr298 presumably has no chance of competing with the correctly positioned catalytic nucleophile Glu358. Since this tyrosine is conserved throughout the family, located in close proximity to the active center and quite likely hydrogen-bonded to the catalytic nucleophile, it was of interest to explore further the role of Tyr298 *in the wild-type enzyme*, by kinetic analysis of a mutant modified at this position. This is all the more important since similar pairings of an active site nucleophile and a tyrosine are seen in the crystal structures of several glycosidases (Jacobsen et al., 1994; Varghese et al., 1994; Wakarchuk et al., 1994).

The most conservative mutation possible, which removes the potentially important phenolic hydroxyl group, is replacement by Phe. The Tyr298Phe mutant enzyme is folded correctly and catalytically active, but with activities some 100–3000-fold lower than wild-type enzyme, depending on the substrate employed. The activity is not due to contamination of a completely inactive mutant by a small amount of wild-type enzyme because (i) the K_m values for most of the substrates are substantially different in the mutant and wild-type enzymes, up to 30-fold lower in one case, (ii) pre-steady-state kinetic studies reveal the release of a full burst of nitrophenolate followed by a slow steady-state release, as would be expected if the mutant is the active species—contaminating wild-type enzyme would release dinitrophenolate continuously, not in a burst—and (iii) both the pH dependence and the secondary deuterium kinetic isotope effects determined for the mutant-catalyzed reaction are substantially different from those of the wild-type enzyme.

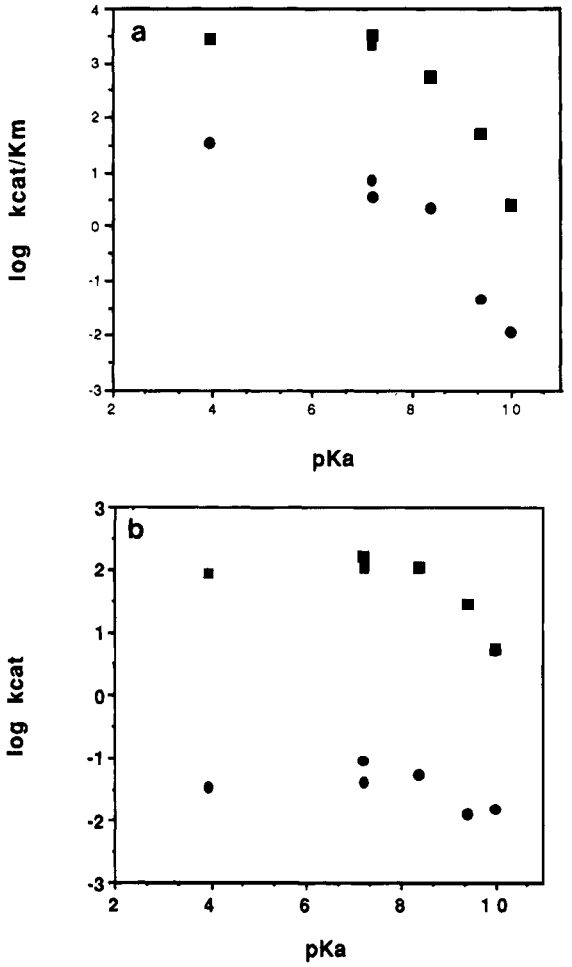

FIGURE 3: Brønsted relationships for the Tyr298Phe and wild type β-glucosidases. (a) A plot of log (k_{cat}/K_m) versus aglycone phenol pK_a for (■) wild-type and (●) Tyr298Phe mutants. (b) A plot of log (k_{cat}) versus aglycon phenol pK_a for (■) wild-type and (●) Tyr298Phe mutants.

Brønsted plots of log (k_{cat}/K_m) vs pK_a for Tyr298Phe and the wild-type enzyme are very similar (Figure 3a). Interestingly, the basis of the curvature in this plot for the wild-type enzyme was thought to be diffusion control of the reaction rate with the best substrates (Kempton & Withers, 1992). However, the similar curvature with the mutant, but at 100-fold lower rates, renders this explanation unlikely. The Brønsted plots of log k_{cat} (Figure 3b) are also quite similar, although there appears to be a less complete change-over to rate-limiting glycosylation with the poorer substrates for Tyr298Phe than for the wild-type enzyme, where the plot has a pronounced biphasic character. The slopes of the leaving group-dependent parts of the log k_{cat}/K_m versus pK_a plots give insight into the degree of negative charge development at the phenolate oxygen. This is best seen in a plot of log (k_{cat}/K_m) for the hydrolysis of all substrates in this range by the wild-type enzyme vs the same data for Tyr298Phe (Figure 4). This plot has a correlation coefficient of $\rho = 0.9$ and a slope of 0.93, suggesting similar transition states, but with slightly less charge development on the phenolate oxygen in the mutant. These results suggest a smaller degree of bond breakage at the transition state for the mutant than for the wild-type, i.e., an earlier transition state.

The removal of the phenolic hydroxyl group of Tyr298 causes considerable reductions in the rates of both the glycosylation step (formation of the glycosyl−enzyme) and

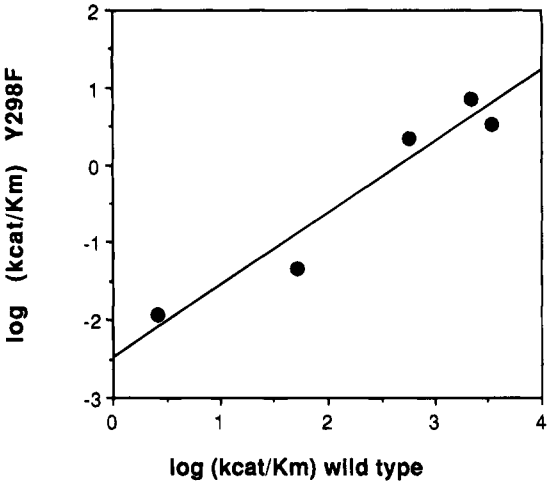

FIGURE 4: Linear free energy relationship comparing the glycosylation steps for the wild-type and Tyr298Phe mutant. A plot of log (k_{cat}/K_m) values for hydrolysis of each of a series of aryl glucosides by the Tyr298Phe mutant versus the equivalent parameter for the wild-type enzyme.

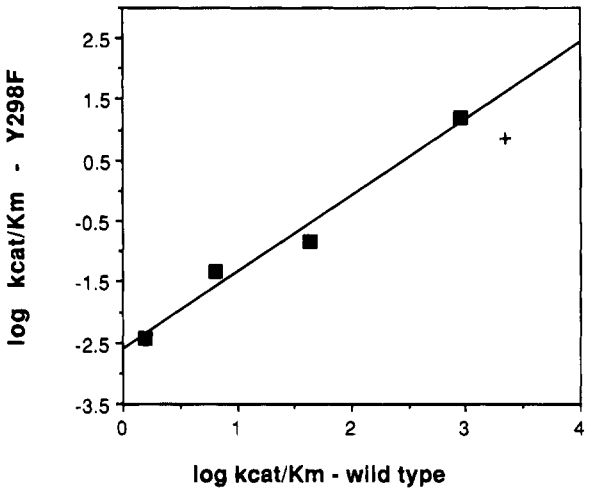

FIGURE 5: Linear free energy relationship relating specificity of the mutant and wild-type enzymes. A plot of log (k_{cat}/K_m) values for hydrolysis of each of a series of substrates differing in their glycon moieties by the Tyr298Phe mutant versus the equivalent parameter for the wild-type enzyme: "+", value for PNPGlu.

of the deglycosylation step (hydrolysis of the glycosyl−enzyme). Comparison of k_{cat} values for the more activated glycosides, for which pre-steady-state analysis indicates that deglycosylation is rate-limiting in both cases, gives a reduction of some 2000-fold in the rate of the deglycosylation step. Comparison of k_{cat} values for phenyl glucoside, for which glycosylation is the rate-limiting step, reveals a 360-fold slowing of the glycosylation step as a consequence of mutation. Similarly, comparison of k_2/K_d values for PNPGlu obtained by stopped-flow analysis of the mutant and wild-type enzymes reveals a 750-fold drop in that case. This relatively greater slowing of the deglycosylation step than the glycosylation is also reflected in the very low K_m values seen for the more activated substrates.

The specificity of the enzyme for the sugar part of the substrate is not significantly changed compared to that of the wild type, rates of hydrolysis of all substrates being reduced some 50−400-fold as measured through k_{cat}/K_m values. This is best expressed in the form of the linear free energy relationship shown in Figure 5. Such plots, in which rates with different substrates are compared, are a measure

of active site homology at the transition state for the first irreversible step, glycosylation of the enzyme (Withers & Rupitz, 1990; Sierks et al., 1992). For a perfectly conserved active site structure at the transition state, such a plot would yield a line with a slope and correlation coefficient of 1. The correlation coefficient of 0.98 and slope of 1.26 observed indicate that removal of the tyrosine hydroxyl does not significantly disturb interactions with any individual sugar hydroxyl groups at the active site since; were this to be the case, points corresponding to the substituted position would lie considerably off the line. Given the location of Tyr298 near the anomeric center, the most likely substituent with which it might interact would be the 2-hydroxyl. However, the point for the 2-deoxy sugar is very well accommodated by the line, suggesting that no significant interactions develop between the 2-hydroxyl and Tyr298 at the first transition state. The slope of the plot (1.26) indicates a greater sensitivity of the transition state for the mutant toward substitution than is seen with the wild-type enzyme. Since this change does not appear to be due to differences in interactions at the hydroxyl groups probed, it may well indicate something more global, such as differences in the position of the transition state along the reaction coordinate, as suggested earlier.

Although these data do not explain the exact role of Tyr298 in catalysis, they clearly establish that this residue is of considerable importance. Perhaps the most likely function is that it hydrogen bonds to the nucleophile, Glu358, thus controlling the ionization state and orientation of this key residue. By such interaction in the free state, it could help to maintain Glu358 in an ionization state which is ready for nucleophilic attack. Upon formation of the glycosyl−enzyme intermediate, this hydrogen bond would be broken, or at least weakened, such that, during the deglycosylation step it could serve essentially as a general acid catalyst, partially donating a proton to the leaving carboxylate and thereby stabilizing it. Equivalent tyrosine residues are indeed found hydrogen-bonded to the nucleophilic residues in several retaining glycosidases for which three-dimensional structures have been determined, suggesting an important role for a tyrosine in this position. Examples include Tyr503 and Glu537 in *E. coli* β-galactosidase (Jacobsen et al., 1994), Tyr69 and Glu78 in *Bacillus subtilis* xylanase (Wakarchuk et al., 1994), and Tyr33, Tyr168, and Glu231 in the β-(1−3)/(1−4) glucanase from *Bacillus sp.* (Varghese et al., 1994). Indeed, a role of Tyr503 in *E. coli* β-galactosidase in some form of protonic catalysis was proposed previously (Loeffler et al., 1979) and received substantial support from studies of mutants modified at that position (Ring et al., 1988).

In the case of the *Agrobacterium* β-glucosidase, the lower rate and altered pH dependence of the Tyr298Phe mutant as compared to the wild-type enzyme would be consistent with the removal of such protonic assistance. The shift in pH optimum for the deglycosylation step could well reflect the removal of a residue which stabilises the departing carboxylate (Glu358) and possibly recruitment of another of higher pK_a. Further, the smaller secondary deuterium kinetic isotope effect measured on the deglycosylation step for the mutant than the wild-type enzyme (k_H/k_D = 1.03 versus k_H/k_D = 1.10) suggests a more S_N2-like reaction. This would be consistent with a poorer enzymic "leaving group" (Glu358) in the absence of stabilization from Tyr298, thereby requiring greater nucleophilic assistance from the water thus a more S_N2-like transition state. Possibly such a catalytic diad will turn out to be a common structural and mechanistic feature of retaining glycosidases as structures of more such enzymes are determined.

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