Identification of the General Acid/Base Catalyst of a Family 3 β-Glucosidase from *Flavobacterium meningosepticum*†

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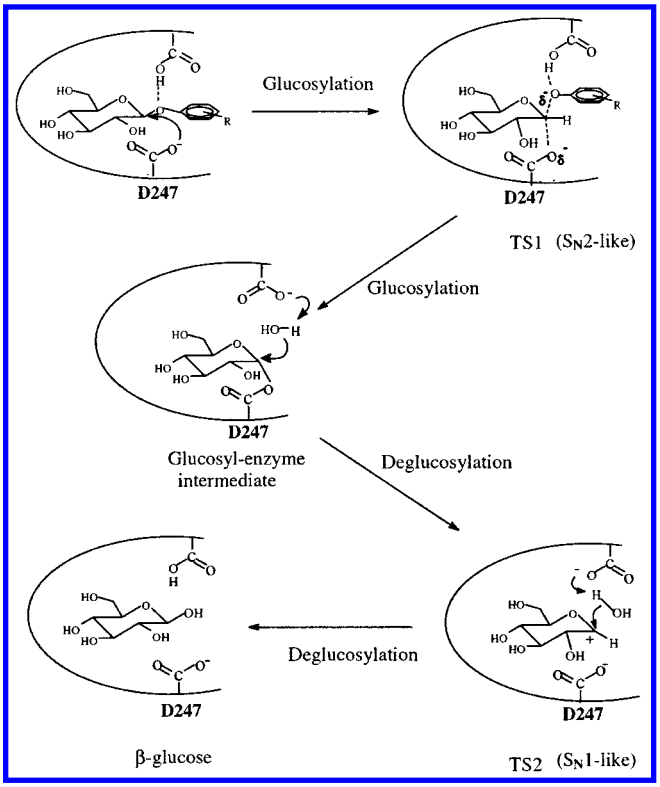
ABSTRACT: β-Glucosidase from *Flavobacterium meningosepticum* (Fbgl) (also known as *Chryseobacterium meningosepticum*) has been classified as a member of the family 3 glycohydrolases. It is a retaining enzyme involving a two-step, double-displacement mechanism. D247 was shown to function as the nucleophile of the enzymatic reaction [Li, Y.-K., Chir, J., and Chen, F.-Y. (2001) *Biochem. J. 355*, 835−840]. However, the general acid/base catalyst of this enzyme and of all other family 3 glycohydrolases has not yet been identified. On the basis of amino acid sequence alignment of 15 family 3 enzymes, 11 residues (D71, R129, E132, E136, D137, K168, H169, E177, D247, D458, and E473) are highly conserved. All of these residues are studied by site-directed mutagenesis and kinetic investigation. Analyzing the catalytic power of all mutants reveals E473 residue is the best candidate of the acid/base catalyst. Detailed studies supporting this suggestion are summarized as follows. (1) The k_{cat} and K_m values for the hydrolysis of 2,4-dinitrophenyl β-D-glucopyranoside (2,4-DNPG) by E473G are reduced 3300- and 900-fold, respectively, compared with those of the wild type (WT). (2) The k_{cat} values of E473G-catalyzed hydrolysis are virtually invariant with pH over the range of 5.0−9.0. (3) The activity of E473G with 2,4-DNPG is enhanced by the addition of azide, and β-glucosyl azide is formed. (4) The k_{cat} of the reaction of 2-carboxyphenyl β-glucoside catalyzed by E473G is comparable to that for hydrolysis by wild-type Fbgl and is 100- and 320-fold better than the k_{cat} values for the E473G-catalyzed hydrolysis of 4-carboxyphenyl β-glucoside and the corresponding methyl ester, respectively. (5) The accumulated glucosyl−enzyme intermediate was directly observed by mass analysis in the reaction of 2,4-DNPG with E473G. All of these results confirm that E473 is the general acid/base catalyst of Fbgl.

On the basis of sequence similarity, glycohydrolases from all sources have been classified into 82 families with some enzymes remaining unclassified. This classification has been best described and demonstrated in a Web page (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). These groups of enzymes cleave the glycosidic bond of the substrate in two different manners: retention and inversion of the anomeric configuration. Inverting glycosidases follow a one-step, single-displacement mechanism with the assistance of a general acid and a general base. The general base polarizes a water molecule to develop a stronger nucleophile for attacking the anomeric carbon, while the general acid protonates the glycosidic oxygen to accelerate the reaction (*1*). Retaining glycosidases are generally believed to catalyze hydrolysis via a two-step, double-displacement mechanism, shown as Scheme 1 (the case of *Flavobacterium* β-glucosidase). Two essential amino acid residues, one functioning as the nucleophile and the other as the general acid/base, are involved. In the first step (glycosylation step), the

Scheme 1: Mechanism of *Flavobacterium* β-Glucosidase



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(a)  69GMDVIHG  127WGRVSEGSGEDPY  167VKHFALYGAPEG  241NGFIVTDY  454ANKADVVVLAIGETAELSGESSS
(b)  46LSDGPTG  114GGRLFEAYSEDPL  148LKHLVANES-ET  222TGLVMSDW  567AAQADVAVVVVGLTEEEETESVD
(c)  43LSDGPNG  111NGRGFESFSEDST  145IKHFVCNDM-ED  219KGTIISDW  559AKSVDCVILCVGLTAEWETEGED
(d)  53VSDGPNG  111GGRGFESFSEDPY  145VKHFVCNDL-ED  219DGMLMSDW  571AAKHDKAVLIIGLNGEWETEGYD
(e)  39VTDGPNG  108NGRNFECYSEDPA  142IKHFVANES-EI  216DGVVMSDW  542ARKSDIVLLLVGREGEWDTEGLD
(f)  42MTDGPHG  117CGRNFEYFPEDPY  151LKHFAANNQ-EH  226DGFVVSDW  401ASSSDVAVVFAGLPDEYESEGFD
(g)  42VSDGPHG  117SGRNFEYFSEDPY  151LKHFAANNQ-EH  225EGIVVSDW  401ALKADVAVIFAGLPEHYECEGYD
(h)  52VSDGPHG  125CGRNFEYFSEDPY  159LKHFAANNQ-EH  233DGLVMSDW  404AMNADKVVVFAGLPDSFESEGFD
(i)  97ETDAGQG  177NGRNFEYAGEDPL  211LKHFVLNDQ-ET  285RGYVMSDW  475AAGADVALVFAN---QWIGEAND
(j)  94ISDAGLG  163GGRNFEYAGEDPL  197LKHYAMNDL-ET  271PGFVMSDW  461ARAADVVVVYAT---QFTFEGMD
(k)  97ETDASLG  166NGRNFEYLGEDPL  200VKHFSLNGQ-ET  274KGWVMSDW  466ARQSDIVILFAN---QWMSEGMD
(l)  81GTDGPAG  144AGRNFETFSEDPL  178AKHYAANTQ-ET  251KGWVMSDW  544ARDSDVAVVFAY---DDGAETAD
(m) 133AYDVVHG  167WGRASEGFGEDTY  207VKHFAAYGAVEG  281KGITVSDH  499AKQADVVVAVVGESQGMAHEASS
(n) 133AYDVLHG  191WGRASEGFGEDTY  231VKHFAAYGAVEG  305KGITVSDH  523AKQSDVVVAVVGEAQGMAHEASS
(o) 110GMDVIHG  168WGRVSEGNGEDPF  208VKHFALYGASEA  282DGFVVTDY  495AAGADVIVAALGESSEMSGESSS
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FIGURE 1: Multialignment of family 3 β-glucosidases. The sequence alignment was achieved using Biology WorkBench 3.2 CLUSTALW provided by San Diego Supercomputer Center (San Diego, CA). The catalytic nucleophile in Fbgl is D247, and the putative acid/base catalyst is E473. Only partial sequences are shown, and the conserved basic and acidic residues are indicated by asterisks. All sequences of enzymes are derived from the corresponding genes, which are indicated by GenBank accession number and the strain of microbes as follows: (a) AF015915 from *F. meningosepticum*, (b) AF005277 from *Cellulomonas biazotea*, (c) AL355920 from *Schizosaccharomyces pombe*, (d) X05918 from *Kluyveromyces fragilis*, (e) M59852 from *Agrobacterium tumefaciens*, (f) X15644 from *Clostridium thermocellum* ATCC 27405, (g) Z94045 from *Clostridium stercorarium*, (h) U92808 from *Ruminococcus albus*, (i) D14068 from *Cellvibrio gilvus*, (j) AB003689 from *Acetobacter xylinus* BPR2001 ATCC13127, (k) AF090429 from *Azospirillum irakense* KBC1, (l) Y14327 from *Saccharopolyspora erythraea*, (m) D86507 from *Salmonella typhimurium* LT2, (n) U00007 from *E. coli* K12/BHB2600, and (o) AF006658 from *Bacteroides fragilis* 638R.

nucleophile attacks at the anomeric carbon of glycoside, whereas the acid/base catalyst protonates the glycosidic oxygen, thereby assisting the leaving of the aglycon moiety. This leads to the formation of a covalent α-glycosyl–enzyme intermediate. In the second step (deglycosylation step), breakdown of the glycosyl enzyme intermediate proceeds through a general base-catalyzed attack of water at the anomeric center to release β-glucose. Both steps proceed through oxocarbenium ion-like transition states. Substantial experimental evidence that supports these mechanisms has accumulated. For more information, see the reviews in refs *1*–*5*. Detailed mechanistic study requires the preparation of a few series of substrates for which the rate-determining step is variable and identifiable, typically through Bronsted plots, pre-steady state kinetics, and kinetic isotope effects. Since enzymes in the same family presumably possess a similar catalytic mechanism, solving the mechanistic details of a particular enzyme may help in the understanding of the reaction pattern of its family. Understanding the mechanism of an enzyme at the molecular level requires knowledge of the active site topology and the essential amino acid(s). To date, less than half of the 82 families were identified on the subject of their essential residues.

β-Glucosidase (EC 3.2.1.21) catalyzes the hydrolysis of the β-1,4-glucosidic bond of variety of glucosides. This group of enzymes has been classified into glycohydrolase families 1 and 3 (*6*, *7*). Enzymes in both families are known to be retaining glycosidases. Some examples of the nucleophile and the general acid/base catalysts, respectively, of family 1 enzymes are E358 (*8*) and E170 (*9*) for *Agrobacterium faecalis* β-glucosidase (Abg), E378 and E206 for *Sulfolobus solfataricus* β-glucosidase (*10*), E383 and E178 for *Streptomyces* sp. β-glucosidase (*11*), and E413 (*12*) and E198 (*13*) for cassava cyanogenic β-glucosidase. However, identification of essential groups of family 3 β-glucosidase needs more informative data and persuasive study. Particularly with regard to the nature of the general acid/base catalyst, no detailed study has been reported so far.

More than 100 family 3 glycohydrolases have been cloned and sequenced. Although all of them bear the common conserved sequences, enzymes within this family can be further classified into six subgroups. Recently, the first X-ray structure of a family 3 enzyme was resolved from barley β-glucosidase (*14*). Unfortunately, this enzyme is very different from Fbgl[1] (this study) in terms of molecular size and phylogenetics. The structure of the barley enzyme provides valuable information about the active site topology, yet it is unable to predict the precise location of the general acid/base catalyst of family 3 enzymes in general. Fifteen enzymes with phylogenetic similarity were chosen and compared. The result of the amino acid sequence alignment is shown in Figure 1. Eleven residues [D71, R129, E132, E136, D137, K168, H169, E177, D247, D458, and E473 (numbering in Fbgl sequence)] are found to be highly conserved. As compared with the sequence of the barley β-glucosidase (data not shown) and the inspected X-ray structure, D95, R158, K206, H207, and D285 in barley enzyme, corresponding to D71, R129, K168, H169, and D247 in Fbgl, respectively, are present in the active site (see Figure 2), whereas E132, E136, D137, E177, D458, and E473, or residues similar to these of Fbgl, are absent in the barley enzyme. D285, in the conserved sequence of SDW, was suggested to be the catalytic nucleophile of the barley enzyme (*14*). This conclusion is consistent with Legler's early study on *Aspergillus wentii* β-glucosidase (*15*) and a recent study on the *Aspergillus niger* enzyme (*16*). Very recently, we also demonstrated that D247 in the TDY sequence serves as the nucleophile of Fbgl (*17*). Our further

[1] Abbreviations: Fbgl, *Flavobacterium meningosepticum* β-glucosidase; ONPG, *o*-nitrophenyl β-D-glucopyranoside; CNPG, 4-chloro-2-nitrophenyl β-D-glucopyranoside; PNPG, *p*-nitrophenyl β-D-glucopyranoside; 2,4-DNPG, 2,4-dinitrophenyl β-D-glucopyranoside; 2,5-DNPG, 2,5-dinitrophenyl β-D-glucopyranoside; 3,4-DNPG, 3,4-dinitrophenyl β-D-glucopyranoside; OCXPG, 2-carboxyphenyl β-D-glucopyranoside; PCXPG, 4-carboxyphenyl β-D-glucopyranoside; MOCXPG, methyl ester of 2-carboxyphenyl β-D-glucopyranoside; MPCXPG, methyl ester of 4-carboxyphenyl β-D-glucopyranoside.

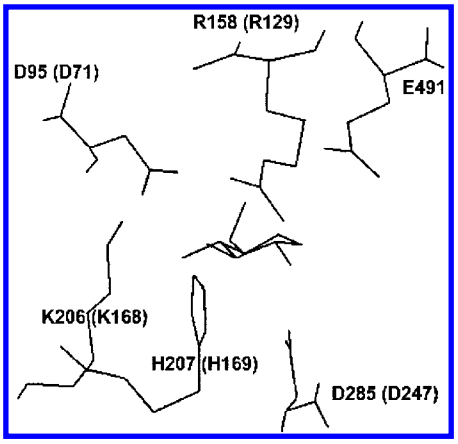


FIGURE 2: Active structure of barley β-glucosidase (1EX1) with glucose incorporated. The corresponding amino acids in Fbgl are labeled in parentheses. The Fbgl structure (not shown) was obtained from the structural simulation based on the sequence homology of barley enzyme and Fbgl with InsightII.

study on labeling Fbgl with 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside and following analysis of the pepsin-digested peptide by tandem mass spectrometry has unequivocally confirmed this point (unpublished data of Y.-K. Li). Although, on the basis of structure, E491 has been suggested to be the general acid/base catalyst of the barley enzyme, it is only present in closely related members of the family 3 enzymes. For those distantly related members, this residue is not conserved (*18*). To identify the acid/base catalyst of Fbgl, all of the 11 conserved residues were mutated and studied by kinetic analysis. This paper presents a detailed investigation of the catalytic role of E473 and turns out to be the first report on the identification of the general acid/base catalyst of the family 3 β-glucosidases.

EXPERIMENTAL PROCEDURES

All microorganisms were obtained from the Culture Collection and Research Center (Hsin-Chu, Taiwan). Buffers were purchased either from Sigma or from E. Merck. Growth medium components were obtained from Difco. Restriction enzymes and DNA polymerase were from New England Biolabs. Oligonucleotides for sequencing and mutagenesis were obtained from a local supplier in Taiwan.

p-Nitrophenyl β-D-glucopyranoside (PNPG) and *o*-nitrophenyl β-D-glucopyranoside (ONPG) were purchased from Sigma Co. The rest of the substrates, except for 2-carboxyphenyl and 4-carboxy β-D-glucopyranosides (*19*), were synthesized from 2,3,4,6-tetra-*O*-acetyl β-D-glucopyranosyl bromide (Aldrich Co.) via aryl 2,3,4,6-tetra-*O*-acetyl β-D-glucopyranoside and deacetylation to form the product (*20*, *21*) and identified by NMR spectroscopy.

Construction of Mutants. Mutagenesis was performed by the Quick Change method (Stratagene Co.), with the following oligonucleotide primers: D71A, 5′-GATTTTTGGAATGGCGGTTATTCATGG-3′ and 5′-CCATGAATAACCGCCATTCCAAAAATC-3′; D71N, 5′-GATTTTTGGAATGAACGTTATTCATGG-3′ and 5′-CCATGAATAACGTTCATTCCAAAAATC-3′; R129K, 5′-CCAAGATGGGGAAAGGTATCGGAAGGT-3′ and 5′-ACCTTCCGATACCTTTCCCCATCTTGG-3′; R129A, 5′-CCAAGATGGGGAGCGGTATCGGAAGGT-3′ and 5′-ACCTTCCGATACCGCTCCCCATCTTGG-3′; E132G, 5′-GGAAGAGTATCGGGTGGTTCTGGTGAGGATCC-3′ and 5′-GGATCCTCACCAGAACCACCCGATACTCTTCC-3′; E136Q, 5′-CGGAAGGATCCGGTCAAGATCCTTATCTGGG-3′ and 5′-CCCAGATAAGGATCTTGACCGGATCCTTCCG-3′; D137D, 5′-CGGAAGGATCCGGTGAAAATCCTTATCTGGG-3′ and 5′-CCCAGATAAGGATTTTCACCGGATCCTTCCG-3′; K168G, 5′-GCTTGCGTAGGGCATTTTGCACTTTATGGTGC-3′ and 5′-GCACCATAAAGTGCAAAATGCCCTACGCAAGC-3′; H169S, 5′-GCTTGCGTAAAAAGCTTCGCACTTTATGGTGC-3′ and 5′-GCACCATAAAGTGCGAAGCTTTTTACGCAAGC-3′; H169A, 5′-GCTTGCGTAAAAGCATTCGCACTTTATGGTGC-3′ and 5′-GCACCATAAAGTGCGAATGCTTTTACGCAAGC-3′; E177Q, 5′-GGTGCACCTCAAGGGGGACGTGATTAC-3′ and 5′-GTAATCACGTCCCCCTTGAGGTGCACC-3′; D247N, 5′-CAATGGCTTTATCGTAACGAACTATACAGGAAT-3′ and 5′-ATTCCTGTATAACACGTTACGATAAAGCCATTG-3′; D275N, 5′-GCAGGTGTTAATATGGATATGGTA-3′ and 5′-TACCATATCCATATTAACACCTGC-3′; D458N, 5′-AATAAAGCAAATGTTGTTGTATTA-3′ and 5′-TAATACAACAACATTTGCTTTATT-3′; E469Q, 5′-GGTGAAACAGCCCAGCTAAGTGGGGAA-3′ and 5′-TTCCCCACTTAGCTGGGCTGTTTCACC-3′; E473Q, 5′-GAACTAAGTGGGCAATCCAGCTCAAGA-3′ and 5′-TCTTGAGCTGGATTGCCCACTTAGTTC-3′; and E473G, 5′-GAACTAAGTGGGGGCTCCAGCTCAAGA-3′ and 5′-TCTTGAGCTGGAGCCCCCACTTAGTTC-3′ (underlining shows the location of the mutation). The template DNA, pCRS1, for mutagenesis was constructed as described in our previous study (*22*). DNA sequence analysis was performed by dye-terminator cycle sequencing on an ABI 310 sequencer.

Protein Purification. Mutant enzymes were produced and purified essentially according to the protocol used for wild-type recombinant Fbgl (*19*). The following procedure, for obtaining the E473G mutant, is typical. All purification steps were performed at ambient temperature (approximately 25 °C). A 1 L culture of *Escherichia coli* XL1-blue carrying pCRS1 cells was grown at 37 °C to midlog phase in LB medium containing 100 mg/L ampicillin. The enzyme was induced by adding IPTG to a final concentration of 0.5 mM, and growth was continued for 10 h. After centrifugation, the cell pellet was resuspended in 10 mL of phosphate buffer (20 mM, pH 7.0) containing 1 mM PMSF. The resulting suspension was sonicated (45 W, 60% pulse) for a total of 10 min with four intervals of 2 min each. The lysate was centrifuged at 10000*g* for 30 min, and the cell free extract was subjected to ammonium sulfate fractionation. The precipitate between 30 and 80% ammonium sulfate saturation was collected and desalted using a HiTrap desalting column (Pharmacia, Uppsala, Sweden) before applying it onto a cation-exchange column (2 × 5 mL, HiTrap SP, Pharmacia) pre-equilibrated with phosphate buffer (20 mM, pH 7.0). The column was eluted with a 60 mL linear gradient of NaCl (from 5 to 200 mM) at a flow rate of 2 mL/min. The purity of the enzyme was analyzed by SDS−PAGE, and the enriched enzyme was pooled and stored at 4 °C for further studies.

Kinetic Studies. Buffers used for this study consisted of 100 mM NaCl and 50 mM buffer: NaOAc (pH 3.5−5.6), MES (pH 5.6−6.5), phosphate (pH 1.0−3.0 and 6.5−7.5), HEPES (pH 6.8−8.2), and Tris (pH 8.0−9.0). Enzyme concentrations were determined as described in a previous

paper (*17*). Rates of enzymatic hydrolysis were measured by incubating the appropriate concentration of substrate in phosphate buffer (50 mM, pH 7.0, 37 °C). Reactions were initiated by adding enzyme, and the reactions were monitored at the appropriate wavelengths. The wavelengths that were employed and molar extinction coefficients ($\Delta\epsilon$, in M^{-1} cm^{-1}) obtained at that wavelength for each glucoside were either measured at pH 7.0 or adapted from the literature (*23*, *24*), and are as follows: 425 nm and 3546 for CNPG, 400 nm and 10 910 for 2,4-DNPG, 440 nm and 4288 for 2,5-DNPG, 400 nm and 11 009 for 3,4-DNPG, 400 nm and 7280 for PNPG, 400 nm and 2170 for ONPG, 315 nm and 1008 for OCXPG, 280 nm and 1780 for PCXPG, 315 nm and 1020 (measured) for MOCXPG, and 280 nm and 1670 (measured) for MPCXPG, respectively. For K_m determination, rates were measured at six to eight different substrate concentrations, ranging from approximately 0.2 times the K_m value determined ultimately to 4−6 times K_m. Very low K_m values for some cases were obtained by paying extra care for measuring the initial velocity. Values of k_{cat} and K_m were calculated by nonlinear regression analysis (*25*). For those cases where K_m values are unusually high (>4 mM), substrates were employed from a low concentration to the concentrations where they can be prepared, and the data were analyzed with a double-reciprocal plot.

Activation of the E473G Mutant with Nucleophiles. The rates of hydrolysis of 2,4-DNPG with a range of different nucleophiles (0−500 mM) were examined at 1 mM 2,4-DNPG ($\gg K_m$), pH 7.0, and 37 °C. The maximum activation rate [$k_{cat(max)}$] was calculated from the plateau value after subtraction of the slow rate of the control reaction (same conditions but without the addition of enzyme). The dependence of k_{cat} on azide concentration for a range of substrates was determined at a single high substrate concentration, which is at least 10 times that of the corresponding K_m values measured in the presence of 200 mM azide.

Mass Spectrometry Analysis. Mass spectra were recorded using a PE-Sciex API triple-quadrupole mass spectrometer. Protein samples were injected into the mass spectrometer via an HPLC system equipped with a PLRP-S column (5 μm, 300 Å, 1 mm × 50 mm) using a 5 to 90% (3 min) acetonitrile gradient system containing 0.1% trifluoroacetic acid as the eluent. Proteins used for molecular weight measurement were normally in the range of 5−10 μg.

RESULTS AND DISCUSSION

Purification and Characterization of Mutant Enzymes. All mutant genes were constructed by the quick change site-directed mutagenesis method. DNA sequencing of the entire coding regions of D71, R129, E132, K168, H169, and E473 mutant clones showed that only the desired single mutations were present. Although other mutants (retaining at least 17% of the wild-type Fbgl activity) were only sequenced in the region with mutated positions, they were inspected by measuring their molecular weights with electrospray ionization mass spectrometry. Molecular weights for all mutants were found to fall in the expected range. Mutants were purified by a procedure similar to that for wild-type Fbgl as described in our previous study (*22*) and in Experimental Procedures. To avoid cross contamination, new columns were used for each mutant. The purity of the enzyme was analyzed by SDS−PAGE. Fractions that were at least 95% homogeneous were pooled and concentrated for further kinetic study. CD spectra of mutants (not shown) were investigated and shown to be virtually identical to that recorded for wild-type Fbgl, indicating no significant perturbation of the secondary structures for all mutants.

Table 1: Michaelis−Menten Parameters for the Hydrolysis of PNPG and 2,4-DNPG by Wild-Type Fbgl and Mutants at pH 7.0 and 37 °C

substrate	enzyme	K_m (mM)	k_{cat} (s^{-1})	$^rk_{cat}$ (%)	k_{cat}/K_m (s^{-1} M^{-1})	$^rk_{cat}/K_m$ (%)
PNPG	wild type	0.49	31.9	100	65100	100
	E132G	30	0.1	0.3	3	0.003
	E136Q	1.08	23.4	73.4	21670	33.3
	D137N	0.71	19.1	59.9	26770	41.0
	H169A	0.48	0.02	0.06	41.7	0.06
	H169S	0.33	0.1	0.31	303	0.47
	D275N	0.79	20.1	62.8	25540	39.2
	D458N	0.92	25.6	80	27830	43
	E469Q	5.15	4.2	13.1	810	1.24
	E473Q	0.49	0.009	0.03	17	0.03
	E473G	0.018	0.006	0.02	333	0.54
2,4-DNPG	wild type	0.36	36.4	100	101050	100
	D71N	0.22	0.43	1.18	1975	1.95
	D71A	4.93	0.015	0.04	3.0	0.003
	R129K	0.71	1.71	4.7	2408	2.35
	R129A	3.47	1.91	5.2	519	0.51
	K168G	0.62	0.12	0.34	199	0.19
	E177Q	0.3	5.15	14.1	17170	17.0
	D247N	1.5	0.0007	0.002	0.5	0.00045
	E473Q	0.34	0.32	0.9	941	0.9
	E473G	0.0004	0.012	0.03	30770	30.5

The stability of the E473G mutant was investigated at various pHs (4.5−9.0) and 37 °C. After incubation for 2 h, the activity was fully maintained in the pH range of 5.0−9.0. However, under acidic conditions (pH ≤4.5), the E473G mutant lost its stability rapidly. It is somewhat more labile than wild-type Fbgl, which is reasonably stable at pH 4.5. This information set a limit for the meaningful interpretation of the pH-dependent kinetic data.

Activities of Mutants. Seventeen mutants were obtained by singly converting 13 amino acid residues in Fbgl into different residues. Their Michaelis−Menten parameters were measured toward 2,4-DNPG (substrate with a good leaving group) and PNPG (with a poorer leaving group). Results are summarized in Table 1. On the basis of the structure simulation (see Figure 2), highly conserved residues D71, R129, K168, H169, and D247 are present in the active site of Fbgl. D247 has been shown to function as the nucleophile of Fbgl. Removing it results in an activity (k_{cat}) loss of $>10^4$-fold (*17*). The catalytic powers of the rest of these active site mutants were reduced some 20−2500-fold. However, the K_m values, except for those of D71A (4.93 mM) and R129A (3.47 mM), were comparable to that of wild-type Fbgl (0.49 and 0.36 mM for PNPG and 2,4-DNPG, respectively). On the basis of the catalytic features of β-glucosidase, the first irreversible step (the glucosylation step) assessed through k_{cat}/K_m measurements largely requires the participation of the general acid/base catalyst when poor substrates are applied. For good substrates, the glycosylation step needs less assistance from the acid/base catalyst. The deglycosylation step, assessed from k_{cat} values of good substrates, is dependent on the assistance of the acid/base catalyst that functions as a general base at this stage. Obviously, if the

general acid/base catalyst is removed, the k_{cat} value will be significantly reduced for all substrates, whereas the perturbation of the k_{cat}/K_m value is very much dependent on the substrate that is employed. When the acid/base mutant catalytically hydrolyzes good substrates such as 2,4-DNPG, the k_{cat}/K_m value should not be affected significantly, yet a very low k_{cat} can be expected since the rate-limiting step is deglycosylation. Such a low k_{cat} value will result in accumulation of the glycosyl−enzyme intermediate and consequently be reflected in a decreased K_m value. These theoretical assumptions have been strongly supported by the studies of *A. faecalis* β-glucosidase (*9*) and *Cellulomonas fimi* exoglycanase (*26*). In this study, as can be seen in Table 1, the kinetic outcomes of E473 mutants seem to match the above descriptions. The K_m value of E473G against 2,4-DNPG is 0.0004 mM, a value 900-fold smaller than that of wild-type Fbgl, and its k_{cat} value is reduced 3300-fold. However, the k_{cat}/K_m (30 770 M^{-1} s^{-1}) exhibits only a 3.3-fold decrease. In the case of hydrolysis of PNPG by E473G, the K_m, k_{cat}, and k_{cat}/K_m were reduced by factors of 27, 5000, and 190, respectively. This is consistent with the results from the Bronsted correlation (log k_{cat} vs pK_a) which show that the rate-limiting step in the hydrolysis of PNPG is at the borderline between glycosylation and deglycosylation (*17*). Other point mutations such as E132G, E136Q, D137N, E177Q, D275N, D458N, and E469Q indicate that these residues that were mutated are unlikely candidates for the general acid/base catalyst, though the catalytic activity (k_{cat}/K_m) of E132G is decreased by 3.3×10^5-fold. However, several observations about catalysis by E132G are inconsistent with Glu132 being the general acid/base catalyst. (1) There is an exceedingly high K_m (30 mM) for PNPG with E132G. (2) There is no significant rate enhancement and no glucosyl azide production observed in the E132G-catalyzed hydrolysis of 2,4-DNPG in the presence of 200 mM sodium azide (see a later section for more detailed discussions). (3) No glucosyl−enzyme intermediate was detected by mass analysis.

pH Dependence. It is common that the catalytic reactivity of many retaining glycohydrolases is mainly mediated by two active site carboxylates (aspartate and/or glutamate). A bell-shaped activity profile reflecting two apparent pK_as is often observed. The first pK_a is normally <5, whereas the second pK_a is in the range of 6.0−7.5. Values of k_{cat} for hydrolysis of 2,4-DNPG by wild-type Fbgl and the E473G mutant as a function of pH within the stability range (4.4−9.2 for the wild type and 5.5−8.8 for E473G) of the enzyme were determined. The pH dependence of k_{cat} of wild-type Fbgl follows a bell-shaped curve with a first apparent pK_a value of <5 and a second pK_a value of approximately 7.3 (Figure 3a). In contrast, no pH dependence was observed for the E473G mutant. Note that, because of the instability of the E473G mutant at pH <5, the anticipated first pK_a was not obtained. The lack of a pH dependence at the higher pH range in E473G catalysis suggests that the group responsible for the pH dependence has been removed.

Rate Enhancement by Exogenous Nucleophiles. For both family 1 and 3 β-glucosidases, the rate-limiting step for hydrolysis of good substrates (pK_a of leaving phenols of <7.0) has been shown to be the deglucosylation step (Scheme 1). As shown above, removal of the general acid/base residue has little effect on the glucosylation step for highly activated substrates, such as 2,4-DNPG, that barely need assistance from a general acid. However, the absence of the acid/base catalyst will make the deglucosylation step even more rate-limiting. Without assistance from the acid/base catalyst, slow hydrolysis of the glucosyl−enzyme intermediate will be expected. This activity loss may be recovered by adding suitable nucleophiles. In fact, hydrolysis of 2,4-DNPG by *Agrobacterium* β-glucosidase (Abg) and the acid/base mutant, E170G, clearly confirmed this prediction. The k_{cat} values obtained with this mutant were largely enhanced (70−300-fold) by adding nucleophiles such as azide and carboxylates (*9*) as 2,4-DNPG was hydrolyzed. This activation somewhat relates to the active site topology. Replacing the acid/base catalyst with the small Gly residue presumably results in sufficient space in the active site so that a small nucleophile can be accommodated in this cavity near the β-face of the glucosyl−enzyme intermediate. Rate enhancement is expected if the small anion functions as a nucleophile or as a general base. In this study, the addition of nucleophiles in the E473G-catalyzed hydrolysis of 2,4-DNPG increases the rate 2−130-fold (Table 2). None of these nucleophiles were found to affect hydrolysis catalyzed by

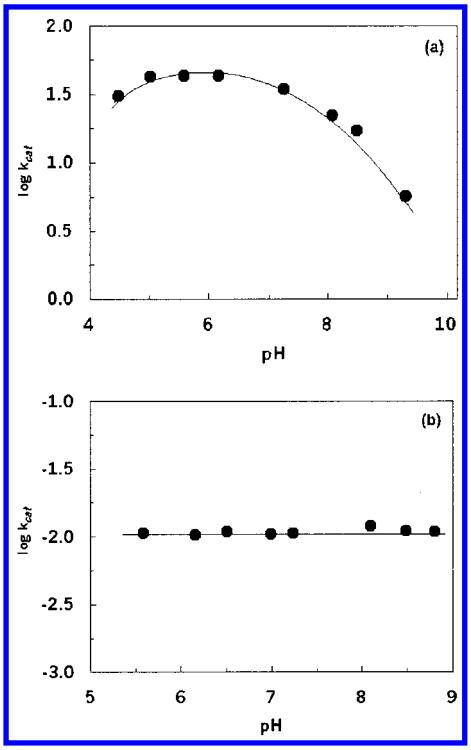


FIGURE 3: pH−activity profile for the hydrolysis of 2,4-DNPG. Plots of log k_{cat} vs pH for the wild type (a) and E473G (b).

Table 2: Apparent k_{cat} Values for the Hydrolysis of 2,4-DNPG by E473G in the Presence of Various Nucleophiles

nucleophile	apparent $k_{cat(max)}$ (s^{-1})	apparent $k_{cat(max)}/k_{cat}$
none	0.01	1
NaOAc	0.02	1.9
$NaHCO_3$	0.14	12
NaN_3	1.57	131
NaS_2O_3	0.17	13.8
HCOONa	0.19	16
DTT	0.20	16.4
2-mercaptoethanol	0.17	14
pyridine	0.04	3.2

Table 3: Michaelis−Menten Parameters for Reactions of Aryl Glucosides Catalyzed by E473G in the Absence and Presence of Sodium Azide

[sodium azide] (mM)	phenol substituent	pK_a	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	log k_{cat}	log k_{cat}/K_m
0	2,4-dinitro	3.96	0.0004	0.012	30.0	−1.92	1.48
	2,5-dinitro	5.15	0.0006	0.013	21.7	−1.89	1.34
	3,4-dinitro	5.36	0.0025	0.018	7.2	−1.74	0.86
	4-chloro-2-nitro	6.45	0.0017	0.008	4.7	−2.10	0.67
	4-nitro	7.18	0.0180	0.006	0.3	−2.22	−0.48
	2-nitro	7.22	0.0040	0.009	2.3	−2.05	0.35
200	2,4-dinitro	3.96	0.02	1.40	70.0	0.146	1.85
	2,5-dinitro	5.15	0.03	0.58	19.3	−0.237	1.28
	3,4-dinitro	5.36	0.07	0.79	11.3	−0.102	1.05
	4-chloro-2-nitro	6.45	0.03	0.32	10.7	−0.491	1.03
	4-nitro	7.18	0.24	0.015	0.06	−1.824	−1.20
	2-nitro	7.22	0.12	0.20	1.7	−0.70	0.22

wild-type Fbgl significantly. Among the tested nucleophiles, azide was most effective. However, interestingly, when the azide reactivation of E473G was monitored by time course NMR spectroscopy (data not shown), both β-glucosyl azide (a doublet at 4.67 ppm, $J = 8.9$ Hz, corresponding to the C1 proton of the β-anomer) and glucose (for the β-form, 4.58 ppm, $J = 7.8$ Hz; for the α-form, 5.20 ppm, $J = 3.3$ Hz) were formed with a ratio of 1:4. This product ratio did not change significantly during a 3 day incubation, indicating that β-glucosyl azide is reasonably stable. This excludes the possibility of forming glucose from subsequent hydrolysis of β-glucosyl azide. Also, no detectable glucosyl azide was observed either during a long-term incubation of 2,4-DNPG and azide or in the mixture of E473G, glucose (10 mM), and azide (200 mM). Clearly, both β-glucosyl azide and β-glucose were produced from the catalytic reaction of 2,4-DNPG. Azide (conjugate acid p$K_a = 4.7$) may function not only as nucleophile but also as a general base. This situation is somewhat similar to the case of the aspartate aminotransferase mutant (K258A), in which catalytic activity was restored by exogenous amines. However, the added species did not act as a nucleophile but as an acid/base catalyst (*27*). Another good example for depicting the general base function of an exogenous molecule was provided by the X-ray crystallographic study of myrosinase, which is an unusual member of the family 1 enzymes lacking the acid/base residue in its active site. A glutamine residue was found to replace the essential glutamate residue of the classical β-glucosidases. L-Ascorbate, a strong activator, is believed to be recruited in the middle of a reaction path to ensure base catalysis (*28*). Alternatively, azide may induce a conformational change in E473G so that more effective recruitment of another residue, which can now serve as a new acid/base catalyst, could occur. This complexity of the azide effect in Fbgl was not observed in the case of family 1 β-glucosidase (*9*, *11*), wherein β-glucosyl azide was the sole product.

Since azide is the strongest activator of E473G, it was chosen for a detailed investigation of rate activation. As shown in Table 3, the k_{cat} and K_m values for the hydrolysis of a range of β-aryl glucosides (pK_a of leaving phenols of 4.0−7.0) by E473G were measured in the absence and presence of azide (200 mM). Azide significantly increased both the k_{cat} (22−116-fold) and K_m (13−50-fold) values for nearly all the tested substrates except PNPG, for which the k_{cat} was only slightly enhanced (2.5-fold). This small rate enhancement indicates that the glucosylation step is, at least

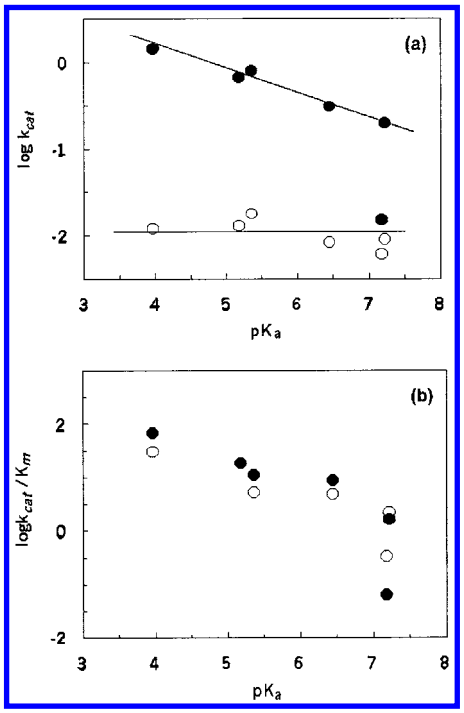
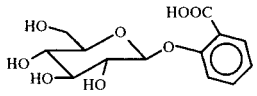
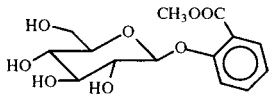
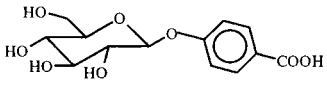
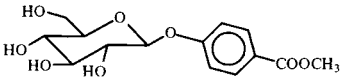


FIGURE 4: Bronsted plot of the hydrolysis of aryl glucosides catalyzed by E473G. Data were taken from Table 3. (a) Plots of log k_{cat} vs pK_a of the aglycon phenol with (●) and without (○) the addition of 200 mM sodium azide. (b) Plots of log k_{cat}/K_m vs pK_a of the aglycon phenol with (●) and without (○) the addition of 200 mM sodium azide.

partially, rate-limiting for the hydrolysis of PNPG by E473G. Bronsted relationships can be obtained by plotting k_{cat} and k_{cat}/K_m values versus the pK_a of the corresponding aglycon phenol (Figure 4). It is not surprising that k_{cat} values are independent of the aglycon pK_a when the reaction is catalyzed by E473G in the absence of azide (Figure 4a), because the deglucosylation step is rate-limiting for all the tested substrates except PNPG. However, in the presence of azide (200 mM), there is a dependence of k_{cat} on leaving group ability, suggesting that the glucosylation step is becoming rate-limiting under such conditions. This is consistent with the observed dependence of k_{cat} on azide concentration (Figure 5). The k_{cat} values approach a plateau at increasing azide concentrations. The value of k_{cat} at this plateau is a function of the pK_a of the leaving group. The observation of a different plateau for each tested substrate provides a strong indication that the plateau is not due to saturation binding of azide to E473G. The curvature seen in

Table 4: Michaelis−Menten Parameters of Carboxyphenyl β-D-Glucosides and Derivatives Catalyzed by Fbgl and E473G[a]

Aryl β-glucosides	Enzyme	Azide (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
	WT	0	30	0.012	0.0004
	E473G	0	0.2	0.032	0.16
	E473G	200	1.85	0.350	0.19
	WT	0	0.12	0.004	0.033
	E473G	0	0.13	0.0001	0.0008
	E473G	200	1.89	0.0002	0.0001
	WT	0	2.2	0.1	0.045
	E473G	0	0.3	0.0003	0.001
	E473G	200	ND	ND	ND
	WT	0	0.72	1.17	1.63
	E473G	0	0.1	0.003	0.03
	E473G	200	4.5	0.014	0.003

[a] ND denotes no significant activity was detected.

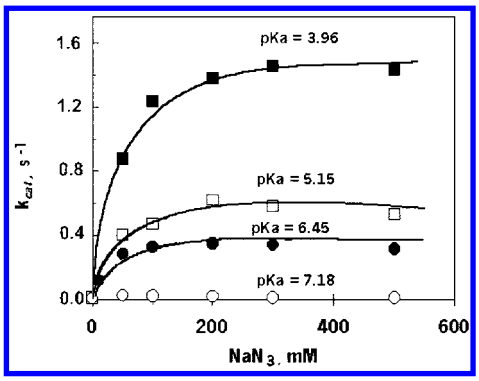


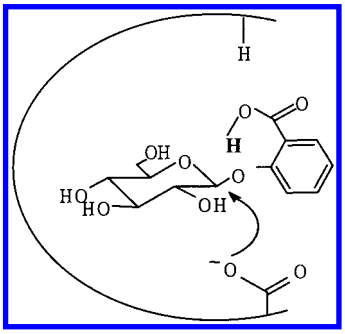
FIGURE 5: Plot of k_{cat} values vs concentration of sodium azide for aryl glucosides catalyzed by E473G: 2,4-DNPG (■), 2,5-DNPG (□), CNPG (●), and PNPG (○).

Figure 5 is most likely due to a change in the rate-limiting step from deglucosylation to glucosylation because the deglucosylation rate is greatly enhanced by azide.

The large increases in K_m values (equivalent to [E][S]/∑[ES]) by adding azide can be easily understood since less glucosyl−enzyme intermediate accumulates. However, as can be seen in Figure 4b, the values of k_{cat}/K_m, reflecting the first irreversible step, are relatively insensitive to the addition of azide, indicating that the azide effect is mainly on the deglucosylation step and not on the glucosylation step. In addition to this study, the azide effect has also been successfully applied to the elucidation of the general acid/base catalyst of many other enzymes in different families (*26*, *29*, *30*).

Substrate-Assisted Catalysis. On the basis of the kinetic analyses for all mutants, E473 is the best candidate for the general acid/base catalyst. Mutation of E473 to Gly slows the glucosylation step of PNPG by 180-fold (calculated from k_{cat}/K_m). For phenyl β-D-glucoside, a relatively poor substrate requiring significant acid assistance, the rate of the glucosylation step was reduced even more dramatically ($>10^4$-fold). Though the glucosylation step for 2,4-DNPG was only slightly affected by the Gly replacement of E473, the rate of the deglucosylation process was reduced by 3000-fold. These results provide a possibility of restoring the activity of the E473G mutant by using a substrate in which a carboxylic acid is incorporated into the structure at an appropriate position to allow donation of a proton at the glycosidic oxygen. For better comparison, 2-carboxyphenyl β-D-glucoside, 4-carboxyphenyl β-D-glucoside, and their corresponding ester derivatives were prepared for this purpose. Results of hydrolyzing these substrates with wild-type Fbgl and E473G with and without the addition of sodium azide are summarized in Table 4. The k_{cat} values for this group of substrates catalyzed by wild-type Fbgl are comparable to, or even smaller than, that for phenyl β-D-glucoside (0.09 s^{-1}), indicating that the rate-limiting step of the reaction is the glycosylation step. This conclusion was

Scheme 2: Intramolecular Proton Donation of 2-Carboxyphenyl β-D-Glucoside



also supported by observing only a small effect of azide (<11-fold rate enhancement) in the E473G-catalyzed reaction. Examining k_{cat} values of both carboxylate substrates catalyzed by E473G revealed that 2-carboxyphenyl β-D-glucoside (OCXPG) is a 100-fold better substrate than the electronically equivalent 4-carboxyphenyl β-D-glucoside (PCXPG). This rate enhancement can be attributed to the existence of the ortho carboxyl group, which promotes the reaction by intramolecular proton donation shown in Scheme 2. Furthermore, by incorporation of this ortho carboxyl group into the substrate, the activity of E473G can be fully recovered. This can be seen from the k_{cat} values of OCXPG hydrolyzed by E473G (0.032 s^{-1}) and wild-type Fbgl (0.012 s^{-1}). For those substrates lacking the assistance from intramolecular protonation, the activity of E473G is 40−390-fold weaker than that of wild-type Fbgl. K_m values for wild-type Fbgl substrates containing a carboxyl group (30 and 2.2 mM for OCXPG and PCXPG, respectively) are abnormally high, which is likely due to the electrostatic repulsion between the substrate carboxyl group and the active site carboxylic residue. Since the repulsion force between the corresponding ester derivatives and Fbgl is weaker or absent, the K_m values remain in the normal range. By comparison, the K_m values of this group of substrates increase 9−45-fold for the reaction with E473G in the presence of azide (200 mM). This K_m perturbation is probably due to some complicating factors, other than the lower extent of glucosyl−enzyme intermediate accumulation, because glucosylation is already rate-limiting in the absence of azide. The same strategy in the substrate-assisted reaction was used in study of *Agrobacterium* β-glucosidase. However, for that case, the rate-limiting step in the hydrolysis of 2-carboxyphenyl β-D-glucoside is deglucosylation (*24*).

Direct Evidence of E473 as the Acid/Base Catalyst. For good substrates, the main function of the general acid/base catalyst is to polarize a water molecule and therefore enhance its nucleophilicity when it attacks the putative glucosyl−enzyme intermediate. Removing the acid/base catalyst will likely lead to an accumulation of the intermediate. This expectation is indeed supported by the observation of a very small K_m value (0.0004 mM) for 2,4-DNPG with E473G. A LC−MS measurement provided "quick" and unequivocal evidence for this. As shown in Figure 6, a new, major protein peak with a MW of 80 090 was observed after the incubation of E473G with 2,4-DNPG for 5 min. The 160 amu increase in MW suggested (within experimental error) that a glucosyl residue was covalently labeled on the enzyme. This result clearly provides strong evidence that (1) E473 is the general acid/base catalyst and (2) as has been observed with the family 1 β-glucosidases, there is a glucosyl−enzyme intermediate in this family 3 β-glucosidase.

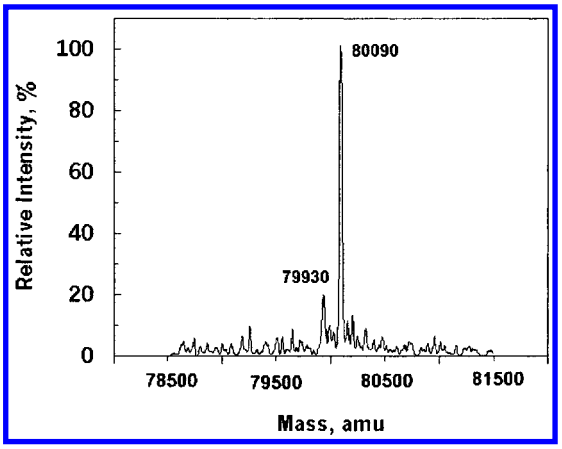


FIGURE 6: Apparent molecular mass of E473G mutant (1.8 μg/μL, 30 μL) after incubation with 2,4-DNPG (20 mM) for 5 min. The smaller peak with a molecular weight of approximately 79 930 amu is the unlabeled E473G mutant. The major peak with a molecular weight of 80 090 amu, 160 amu greater than that of E473G, is believed to be the labeled enzyme.

CONCLUSION

Although the general mechanism of action of the retaining β-glucosidases was proposed by Koshland decades ago (*31*), the formation of a glucosyl−enzyme intermediate in the catalytic path was unequivocally confirmed by the application of 2-fluoro-2-deoxy-β-D-glucosides in the 1990s. This two-step mechanism, for both family 1 and family 3 enzymes, was mediated by two essential residues, which act as a nucleophile and an acid/base catalyst. Many studies have successfully identified both catalytic residues of β-glucosidase by site-directed mutagenesis and kinetics. However, nearly all of them were targeted on family 1 enzymes. In this study, we provide direct evidence for the existence of a glucosyl−enzyme intermediate in a family 3 β-glucosidase as well as the results of several kinetic and site-directed mutagenesis investigations identifying Glu473 as the general acid/base catalyst. This turns out to be the first extensive study on this subject for family 3 enzymes. Although this family can be further classified into six subfamilies (*18*), and the general acid/base catalyst is obviously not fully conserved, this study offers a valuable starting point for locating the acid/base catalyst of family 3 enzymes with phylogenetics similar to those of Fbgl. Also, compared with the numbering domain of family 1 β-glucosidases (higher numeric order for the nucleophile, e.g., E358 in Abg, and lower numeric order for the acid/base catalyst, e.g., E170 in Abg), the acid/base catalyst of Fbgl (a family 3 enzyme) comes after its nucleophile in the linear sequence of amino acids. This case of numbering order is also seen in another family 3 enzyme, such as barley β-glucosidase.

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