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Identifying the Catalytic Acid/Base in GH29 α-L-Fucosidase Subfamilies

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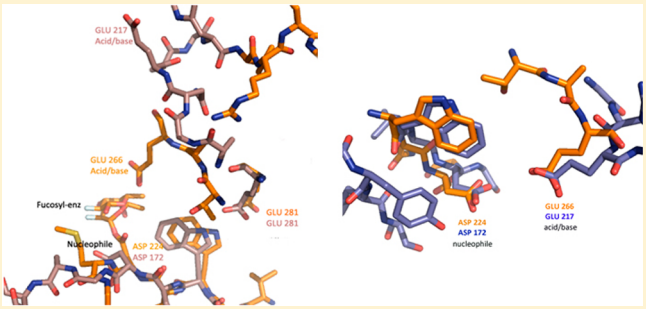
Supporting Information

ABSTRACT: While the catalytic nucleophile in the configuration-retaining α-L-fucosidases from family GH29 is fully conserved with respect to sequence, there is no fully sequence-conserved acid/base residue candidate across the family. X-ray crystallographic studies and kinetic characterizations have allowed the identification of this residue in a few cases, and a recent combination of phylogenetic tree analyses with substrate specificity data has allowed the division of GH29 enzymes into two subfamilies, A and B, allowing the probable assignment of these residues. Here, we perform detailed kinetic and mechanistic characterizations of the corresponding alanine mutants and other candidates. Through comparison of kinetic parameters obtained for the hydrolysis of fucosyl substrates with activated leaving groups by these mutants with those of the corresponding wild-type enzymes, in conjunction with the demonstration of azide rescue, we largely confirm the acid/base residue predictions for the GH29 fucosidases from the two subfamilies.



L-Fucosylated glycoconjugates are widely found in mammalian tissues such as the liver, brain, and spleen as well as on red blood cells as ABO blood group and Lewis antigens.[1−3] Fucosylated glycans are, not surprisingly, heavily involved in many physiological processes, including antigenicity and immune responses, signal transduction, and the adhesion processes in pathogens.[3−7] GH29 α-L-fucosidases are exoglycosidases that most commonly hydrolyze α-(1,2) linkages from fucose to galactose or α-(1,3), α-(1,4), and α-(1,6) linkages to *N*-acetylglucosamine residues.[7−12] Recent results suggest that GH29 fucosidases can be divided into two main subfamilies (A and B) on the basis of phylogenetic relationships and substrate specificities.[13] Subfamily A contains fucosidases that have relatively relaxed substrate specificities, whereas fucosidases from subfamily B are more specific for α-(1,4) or α-(1,3) linkages. Inspection of the active sites of representative enzymes from each subfamily provided an attractive explanation for these specificity differences.[11] Table 1 provides a summary of the fucosidases for which structural and/or detailed mechanistic characterizations are available, along with assignments to subfamilies and principal potential acid/base residues.

GH29 fucosidases have been shown to perform hydrolysis with net retention of configuration at the anomeric center; thus, they are retaining enzymes.[8,14−16] Retaining glycosidases typically use a double-displacement mechanism involving two catalytic carboxylic acids at the active site: a nucleophile and an acid/base residue.[17,18] The catalytic nucleophile in GH29 fucosidases was initially determined to be an aspartate residue [D224 in *Thermotoga maritima* fucosidase (Tmfuc)] through mass spectrometric analysis of a proteolytic digest of a fucosidase inactivated by the mechanism-based covalent inactivator, 2-deoxy-2-fluoro-α-L-fucosyl fluoride.[16] Sulzenbacher et al. later published the crystal structure of this same fucosidase from *T. maritima*, free and as its covalent intermediate, along with kinetic data for the wild type and mutants, all of which supported the assignment of D224 as the nucleophile.[25] This residue is fully conserved across both subfamilies of GH29.

The assignment of the catalytic acid/base residue has been more problematic because the residue initially assigned on the basis of the *T. maritima* structure (E266 in that case) is not fully conserved across the family, necessitating application of experimental approaches. One such approach requires kinetic analysis with substrates with different leaving group abilities. Because the catalytic acid/base residue provides protonic assistance to the departing aglycone, substrates with poor leaving groups are cleaved more slowly by mutants modified at that position than are substrates with good leaving groups. This is a result of the slowing of the glycosylation step of the hydrolysis reaction and is reflected in the second-order rate constant (k_{cat}/K_M).[18−20] For substrates with activated leaving groups, formation of the glycosyl−enzyme intermediate proceeds at rates similar to that of the wild-type enzyme as the leaving group does not require protonic assistance, while

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Table 1. GH29 Fucosidases for Which Crystal Structures and/or Kinetic Characterizations Are Available[a]

species[b]	subfamily	nucleophile	acid/base 1 (structurally aligns with Tmfuc)	acid/base 2 (aligns with Hfuc)	source	ref
			Crystal Structures Available			
BT2192	B	D188	E234	E275	bacterial	PDB entry 3EYP
BT3798	B	D199	E240	none	bacterial	PDB entry 3GZA
BI2336	B	D172	E217	E281	bacterial	11, 13
BT2970	A	D229	E288	E306	bacterial	27
			Have Crystal Structures and Have Been Kinetically Characterized			
Thermotoga maritima	A	D224	E266	E281	bacterial	16, 25
			Kinetically Characterized and No Available Crystal Structure			
Sulfolobus solfataricus	A	D242	E292	none	archaea	8, 31, 32
Homo sapiens A1	A	D225	none	E289	eukaryote	14

[a]The conserved nucleophile and two candidate acid/base positions are listed; the first acid/base position aligns with the acid/base of Tmfuc, and the second potential acid/base position aligns with the acid/base of Hfuc. The listing of kinetically characterized fucosidases does not include the characterizations of the enzymes studied in this work. [b]Species are as follows: BT2192, *Bacteroides thetaiotaomicron* 2192; BT2970, *B. thetaiotaomicron* 2970; BT3798, *B. thetaiotaomicron* 3798; BI2336, *Bifidobacterium infantalis* sp. 2336.

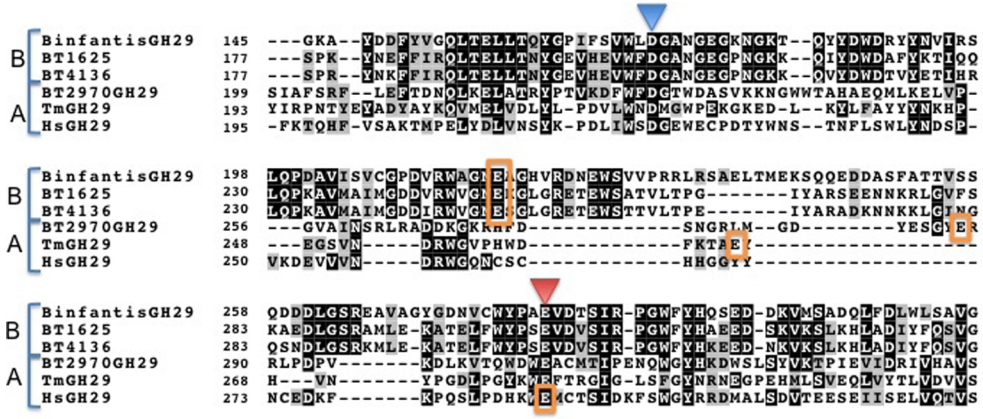

Figure 1. Alignment of representative sequences from subfamilies A and B. The blue arrowhead denotes the conserved nucelophile. Orange boxes indicate experimentally confirmed acid/base residues. The red arrowhead denotes the fully conserved residue at the position of the acid/base for Hfuc.

hydrolysis of the intermediate remains slow because of the lack of base catalysis. As a result, the glycosyl−enzyme intermediate accumulates. This accumulation can be seen as an initial burst of release of the activated leaving group if sufficient enzyme is employed and is also reflected in a low K_M value.[19,20] A second method of identifying the catalytic acid/base residue involves nucleophilic small molecule rescue of activity in mutants lacking their acid/base catalyst. Inclusion of a nucleophilic anion such as azide in reaction mixtures containing an acid/base mutant and a substrate with a good leaving group should accelerate turnover because the azide binds in the vacant site and reacts with the glycosyl−enzyme intermediate more rapidly than water, forming a glycosyl azide product.[21−24]

Such approaches, including chemical rescue studies with mutants, were applied to the human α-fucosidase (Hfuc) by Liu et al. after they had realized that the acid/base residue was not conserved between Hfuc and Tmfuc, despite both being members of GH29.[14] On the basis of such studies, they assigned E289 as the acid/base mutant in the human enzyme. This residue aligns with E281 in Tmfuc, outside the active site, despite the fact that both are now assigned to subfamily A. More recently, Sakurama et al. have performed structural studies of *Bifidobacterium infantis* fucosidase (BiAfcB), a member of subfamily B for which sequence comparison also did not reveal the identity of the acid/base residue.[11] Their studies provided a nice explanation of the substrate specificities in these subfamilies. They also showed that a conformational change occurs upon substrate binding, positioning an otherwise distant carboxylic acid group an appropriate distance from the nucleophile residue for catalysis to occur. However, assignment of these residues as the acid/base catalysts in each case is primarily based upon structures wherein the location of catalytic residues seems to depend upon ligand binding states, and not upon detailed mechanistic analyses. Figure 1 provides an alignment of representative sequences from subfamilies A and B for which experimental verification of the acid/base identity has been achieved (some in this work). The position of the fully conserved glutamic acid that has been shown to function as the acid/base in Hfuc is indicated with a red arrow. This clearly exemplifies the challenges faced in this assignment.

The aims of this paper are to gain further insight into this conundrum concerning acid/base identities in GH29 by experimentally verifying the predicted acid/base residue in several *Bacteroides thetaiotaomicrometer* (BT) GH29 α-L-fucosidases from subfamilies A and B, along with a brief verification of the identity of their nucleophile residues.

EXPERIMENTAL PROCEDURES

Chemicals and Substrates. *p*-Nitrophenyl α-L-fucoside (pNPαFuc) was purchased from Sigma-Aldrich. All reagents were purchased from commercial chemical suppliers (Sigma-Aldrich, Fluka, and Cambridge Isotope Laboratories) and were

used without further purification. α-Fucosyl fluoride was synthesized according to previously published procedures.[26]

Cloning and Mutagenesis. The gene encoding Tmfuc was amplified by polymerase chain reaction (PCR) from genomic *T. maritima* DNA (ATCC 43589) using primers containing restriction sites NdeI and XhoI as follows: Tmfuc-fw (5′-GCC CAT ATG ATT TCT ATG AAA CCC CG-3′) and Tmfuc-rv (5′-GCC TCG AGT TAC TTA ATT ACA ATA TC-3′). Standard PCR conditions were used for 35 cycles with Pfu polymerase, and the 1.3 kb insert was digested (NdeI and XhoI) and ligated into a pET-22b vector similarly digested and also treated with shrimp alkaline phosphatase. Plasmids obtained from selected colonies were submitted for sequencing to the Nucleic Acids and Peptides Service (NAPS) unit at UBC, and plasmids containing the full-length gene with no errors were used in subsequent experiments.

The site-directed mutagenesis by overlap extension (SOE) method was used to generate all Tmfuc mutants (D224A, E266A, and E281A). For the D224A mutant, pET-22bWT was used as the template and two separate PCR mixtures containing the following primer combinations were used in the first amplifications: D224Afw, 5′-CCC GAC GTT CTC TGG AAC GCC ATG-3′ with T7term, and D224Arev, 5′-CCT TCC TCC GGC CAG CCC ATG GCG-3′ with T7. The amplified fragments from this were used as templates in the second PCR using T7 and the T7term primers. This generated a 1.3 kb full-length gene, which was then subcloned into the pET-22b vector using the NdeI and XhoI restriction sites. A similar protocol was followed with D453A. Table 1 lists all the primers used for generating the Tmfuc mutants (along with all BT mutants).

A plasmid encoding BT2970 was produced as described previously.[27] Plasmids containing the D229G mutation and the E288Q mutation were generated using the QuickChange site-directed mutagenesis method as described previously.[28] The wild-type plasmid (BT2970WT) was regenerated by QuikChange site-directed mutagenesis of the D229G plasmid with the following primers: BT2970WTfw, 5′-CCG ACC GTT AAG GAC TTC TGG-3′; and BT2970WTrev, 5′-CCA GAA GTC CTT AAC GGT CGG-3′. The QuikChange method was used to generate the E288A and E306A mutants using the forward and reverse primers listed in Table 1.

Wild-type genes for both BT1625 and BT4136 α-L-fucosidases were amplified by PCR from *B. thetaiotaomicron* VPI-5482 genomic DNA using primers that gave ligation-independent cloning (LIC) compatible ends (BT1625: BT1625Fwd, 5′-CCA GGG ACC AGC AAT GAA CAA ACT ACT TAC TTC TCT CTT TTT ATC TTC-3′; and BT1625Rev, 5′-GAG GAG AAG GCG CGT CAA GGA GCT ACG GTC ACT CCG ATT TC-3′. BT4136: BT4136Fwd, 5′-CCA GGG ACC AGC AAT GAA AGA AAA CTA TTA TGT AAA ACA CGT AGA GTT TC-3′; and BT4136Rev, 5′-GAG GAG AAG GCG CGT CAA GGG GCT ATT GTT ACT CCG ATT TC-3′). The PCR product was ligated into a LIC-modified pET28 vector using previously described methods.[29] The QuikChange method was used to generate the E286A, E294A, E305A, D307A, and E249A mutants (acid/base candidates) using the forward and reverse primer combinations found in Table 1.

Enzyme Preparation. All enzymes were expressed in *Escherichia coli* BL21(DE3) cells, which had been transformed by electroporation using the Genepulser II (Bio-Rad Inc.). For the production of each enzyme, a single colony was inoculated into 50 mL of LB medium containing 100 μg/mL ampicillin (or kanamycin) and grown overnight. From this overnight culture, 5 mL was inoculated into 1 L of LB medium containing 100 μg/mL ampicillin (or kanamycin). Cell cultures were induced with 0.5 mM IPTG once they reached an OD_{600} of 0.7 and then centrifuged at 5000*g* for 20 min after being induced for 4 h (or overnight in some cases). The cell pellets were resuspended in 50 mL of lysis buffer [20 mM Tris (pH 7.9), 0.5 M NaCl, and 20 mM imidazole] and lysed using a French press. EDTA-free protease inhibitor was added after the lysis step, and the lysed cell mixture was centrifuged at 15000 rpm for 30 min. The filtered supernatant was added to a 1.0 mL (or 5.0 mL) HisTrapFF (GE) column and eluted using a gradient from 0.05 to 0.30 M imidazole. Unbound proteins were washed using washing buffer (containing 0.05 M imidazole), and the elution of the desired protein was visualized as a defined peak on a chromatogram following the UV absorbance at A_{280}. Fractions containing the enzyme of interest were concentrated using a 10K or 30K molecular weight cutoff centrifugal filter (Amicon Ultra, Millipore) and dialyzed against a suitable buffer overnight. All enzymes were concentrated and run on sodium dodecyl sulfate−polyacrylamide gel electrophoresis gels to assess their purity, and the approximate molecular weights were determined by comparison to prestained molecular weight markers (Bio-Rad). Repeat column purification was performed if the enzymes were not >95% pure. Fresh columns were used for each mutant to avoid cross-contamination. The enzymes were concentrated, buffer-exchanged into appropriate buffers, and stored at 4 °C. The Bradford assay was used for the determination of protein concentrations.

Enzyme Kinetics. All experiments were conducted at 37 °C using a continuous spectrophotometric assay on a Cary-4000 spectrometer (Varian Inc.) connected to a circulating water bath. Prewarmed 200 μL cuvettes (path length of 1 cm) were loaded with an appropriate volume of water, buffer, and substrate at concentrations from 0.1 to 5 times the K_M and incubated for 5 min before data were acquired. Spontaneous hydrolysis of substrates was monitored prior to the addition of enzyme. Michaelis−Menten parameters for hydrolysis of pNPαfuc were measured by monitoring the release of *p*-nitrophenyl at 400 nm (or 360 nm for Tmfuc). For Tmfuc, kinetic parameters were obtained in the following buffer: 50 mM sodium citrate containing 150 mM sodium chloride and 0.1% BSA (pH 5.0). An extinction coefficient (A_{360}) of 1816 M^{-1} cm^{-1} was determined for *p*-nitrophenol under these conditions. The buffer used for all *B. thetaiotaomicron* fucosidases was 50 mM sodium phosphate buffer (pH 6.5) containing 150 mM sodium chloride and 0.1% BSA. An extinction coefficient of 7450 M^{-1} cm^{-1} was determined for *p*-nitrophenol under these conditions. Kinetic parameters were evaluated by fitting data using GraFit version 4.0 (Erithacus Software).

Rates of hydrolysis of αFucF by Tmfuc, BT2970fuc, BT4136fuc, BT1625fuc, and mutants were determined by following the increase in fluoride ion concentration using the Orion 96-09 combination fluoride ion electrode connected to a computer running LoggerPro (Vernier Software Ltd.). Glass vials containing various concentrations of αFucF were incubated at 25 or 37 °C to establish a steady-state spontaneous hydrolysis rate before the enzyme was added (to a final concentration of 750 μL). Background hydrolysis-corrected initial rates were used to determine the kinetic parameters, which were obtained by direct fit of the data in GraFit version 4.0 (Erithacus Software). TLC analysis of the azide reaction

products was performed on aluminum-backed silica plates using a 7:2:1 ethyl acetate/methanol/water mixture as the eluent.

RESULTS AND DISCUSSION

To gain insight into the identities of potential acid/base residues in subfamilies A and B, the *B. thetaiotaomicron* BT2970 and *T. maritima* Tmfuc fucosidases were selected as representatives from subfamily A, along with two fucosidases from *B. thetaiotaomicron* (BT1625 and BT4136) as representatives of subfamily B. A series of mutants of candidates for the acid/base residue in each case were prepared and subjected to detailed kinetic and mechanistic analysis. Mutations were generally introduced using the QuickChange method except in the case of Tmfuc, where overlap extension was more successful. Enzymes were purified as six-His tag fusions.

Kinetic Characterization of Subfamily A Enzymes BT2970fuc and Tmfuc and Their Mutants. The availability of the structures of both enzymes and the knowledge that subfamily A also includes the human fucosidase suggest two possible candidates for acid catalysts in this case for which alanine mutants were made: those corresponding to the residue seen in the known structures (E266 in Tmfuc and E288 in BT2970) along with the residue corresponding to Hfuc (E306 in BT2970). The structural relationship of these residues is shown in Figure 2.

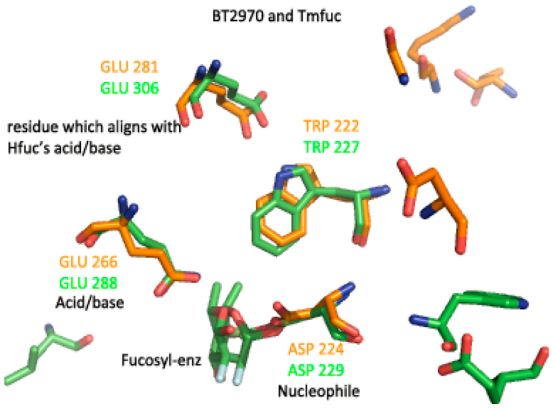

Figure 2. Active site structures of members of subfamily A: BT2970 overlaid on the 2-fluorofucosyl enzyme of *T. maritima* α-L-fucosidase. Only selected active site residues are shown; the structure of Tmfuc is shown with an orange backbone and the structure of BT2970 with a green backbone. The colors of the residues correspond to the colors of the structures.

Kinetic parameters obtained for the hydrolysis of pNPαFuc and αFucF by BT2970 and its E288A (putative acid/base) mutant suggest that deglycosylation is again rate-limiting in both cases because the k_{cat} values for the hydrolysis of the two substrates by the wild-type enzyme are similar to each other while k_{cat} values for the hydrolysis of the two substrates by E288A are also similar to each other, and approximately 2000-fold lower than for the parent. No hydrolysis of pNPαFuc by the D229A (nucleophile) mutant was seen, even up to protein concentrations of 150 μg/100 μL of reaction mixture, highlighting the importance of this residue in catalysis. The k_{cat}/K_M value for hydrolysis of pNPαFuc by the E288A (acid/base) mutant of BT2970 is 25000-fold lower than that of the wild-type enzyme, consistent with the loss of acid/base assistance (Tables 2 and 3). By contrast, the k_{cat}/K_M value for the hydrolysis of αFucF by the E288A mutant was only 35-fold lower than that of the wild type; such a difference in the ratio of k_{cat}/K_M values (wild type to acid/base mutant) between the two substrates is expected with the acid/base mutant because this parameter reflects the rate of the first irreversible step (glycosylation): substrates with poor leaving groups are cleaved more slowly by mutants modified at the acid/base position than are substrates with good leaving groups,[22] providing support for E288 being the acid/base residue. Additionally, the K_M observed for the hydrolysis of αFucF by the E288A mutant was 50-fold lower than that of the wild type, because of the expected accumulation of the intermediate with a substrate that does not require acid catalysis for its glycosylation step. The realization, since then, that two subfamilies exist and that BT2970fuc and the human fucosidases (Hfuc) both belong to subfamily A raises the question of whether the residue in BT2970fuc that aligns with the residue assigned as the acid/base in Hfuc (E306 in BT2970fuc and E289 in Hfuc) plays an important role in those enzymes.

The alanine mutant modified at the corresponding residue (E306A) hydrolyzed pNPαFuc as efficiently as wild-type BT2970, suggesting that this residue does not play a role in catalysis or binding.

Earlier results of Tarling et al. had provided supportive evidence of the identities of the acid/base and nucleophile in Tmfuc, as follows. Kinetic parameters measured for the hydrolysis of 4-nitrophenyl α-fucoside (pNPαFuc) and α-fucosyl fluoride (αFucF) by Tmfuc and its E266A (acid/base) mutant suggested that deglycosylation is again rate-limiting for the wild-type enzyme, because the k_{cat} values for the hydrolysis of the two substrates by the wild-type enzyme are similar and

Table 2. Kinetic Parameters for the Hydrolysis of pNPαfuc and αFucF by the Subfamily A Fucosidases Tmfuc (WT and mutants) and BT2970fuc (WT and mutants)

	pNPαfuc			αFucF		
	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
			BT2970			
wild type	32 ± 2	2.6 ± 0.5	12 ± 3	29 ± 2	7.6 ± 0.9	3.9 ± 0.3
D229A (nucleophile)	NA[a]	NA[a]	NA[a]	–	–	–
E288A (acid/base)	0.018 ± 0.002	39 ± 3	(4.7 ± 0.6) × 10^{-4}	0.016 ± 0.002	0.15 ± 0.05	0.11 ± 0.02
E306A	31 ± 4	3.2 ± 0.6	10 ± 3	–	–	–
			Tmfuc			
wild type	756 ± 28	0.55 ± 0.03	(1.4 ± 0.4) × 10^3	456 ± 11	0.15 ± 0.05	(3.0 ± 0.4) × 10^3
E281A	720 ± 19	0.68 ± 0.04	(1.1 ± 0.2) × 10^3			

[a]No activity observed at 150 μg of enzyme added per 100 μL of reaction mixture.

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Table 3. Kinetic Parameters for the Hydrolysis of pNPαFuc and αFucF by Subfamily B Fucosidases BT4136 and BT1625 and Their Mutants

	pNPαFuc			αFucF		
enzyme	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
BT4136 wild type	27 ± 2	4.5 ± 0.4	5.9 ± 0.7	24 ± 3	6.2 ± 0.5	3.9 ± 0.2
D286A	26 ± 3	5.6 ± 0.6	4.6 ± 0.5	–	–	–
E305A	25 ± 1	2.6 ± 0.3	9.7 ± 0.6	–	–	–
E298A	27 ± 3	8.4 ± 0.7	3.3 ± 0.2	–	–	–
D307A	4 ± 0.4	3.3 ± 0.2	1.1 ± 0.1	–	–	–
E294A	NE[a]	NE[a]	–	–	–	–
D204A (nuc)	NA[b]	NA[b]	NA[b]	–	–	–
E249A (acid/base)	0.041 ± 0.004	23 ± 3	(1.8 ± 0.4) × 10^{-3}	0.039 ± 0.002	0.17 ± 0.02	0.22 ± 0.03
BT1625 wild type	22 ± 2	3.2 ± 0.7	6.8 ± 0.2	21 ± 4	6.4 ± 0.8	3.3 ± 0.1
D286A	12 ± 1	6.4 ± 0.6	1.8 ± 0.2	–	–	–
E305A	10 ± 1	2.7 ± 0.3	3.8 ± 0.3	–	–	–
E298A	12 ± 2	7.6 ± 0.8	1.5 ± 0.1	–	–	–
D307A	1.6 ± 0.3	4.1 ± 0.3	0.40 ± 0.05	–	–	–
E294A	NE[a]	NE[a]	–	–	–	–
D204A (nuc)	NA[b]	NA[b]	NA[b]	–	–	–
E249A (acid/base)	0.023 ± 0.003	36 ± 4	(6.4 ± 0.1) × 10^{-4}	0.021 ± 0.001	0.092 ± 0.006	0.22 ± 0.03

[a]Not expressible. The protein is not expressed under a variety of expression conditions. [b]No observed activity at up to 150 μg of protein added per 100 μL of reaction mixture (protein precipitates at higher concentrations).

Table 4. Kinetic Parameters for the Cleavage of pNPαFuc by Mutants in the Presence and Absence of 200 mM Azide

enzyme	exogenous nucleophile	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (mM^{-1} min^{-1})
BT2970 E288A$^{(acid/base)}$	none	0.018 ± 0.002	39 ± 3	(4.7 ± 0.6) × 10^{-4}
BT2970 E288A	azide	0.063 ± 0.004	72 ± 2	(8.7 ± 0.3) × 10^{-4}
BT2970 D229A$^{(nuc)}$	none	NA[a]	NA[a]	NA[a]
BT2970 D229A	azide	0.016 ± 0.004	3.1 ± 0.4	(5.2 ± 0.6) × 10^{-3}
BT4136 E249A$^{(acid/base)}$	none	0.041 ± 0.004	23 ± 3	(1.8 ± 0.4) × 10^{-3}
BT4136 E249A	azide	0.18 ± 0.2	63 ± 5	(2.8 ± 0.2) × 10^{-3}
BT4136 D204A$^{(nuc)}$	none	NA[a]	NA[a]	NA[a]
BT4136 D204A	azide	0.025 ± 0.002	5.2 ± 0.2	(4.8 ± 0.4) × 10^{-3}
BT1625 E249A$^{(acid/base)}$	none	0.023 ± 0.003	36 ± 4	(6.4 ± 0.1) × 10^{-4}
BT1625 E249A	azide	0.10 ± 0.01	74 ± 8	(1.3 ± 0.2) × 10^{-3}
BT1625 D204A$^{(nuc)}$	none	NA[a]	NA[a]	NA[a]
BT1625 D204A	azide	0.006 ± 0.001	5.2 ± 0.3	(1.1 ± 0.2) × 10^{-3}

[a]No observed activity at up to 150 μg of protein added.

K_M values are quite low in both cases. Deglycosylation remains rate-limiting for the acid/base mutant with αFucF as the substrate, based on its even lower K_M value, while the situation for pNPαFuc is less clear, consistent with the weaker leaving group ability of pNP and an acid/base role for E266.[9,25] Further, the k_{cat}/K_M value for the hydrolysis of pNPαFuc by the acid/base mutant, E266A, was 60000-fold lower than that of the wild-type enzyme, whereas the k_{cat}/K_M value for the hydrolysis of αFucF by the E266A mutant was only 30-fold lower than that of the wild type. Such a difference in the ratio of k_{cat}/K_M values (wild type to acid/base) between the two substrates is consistent with E266 being the acid/base, as explained previously. The role of D224 as the nucleophile was demonstrated through trapping experiments and further supported by the demonstration that the k_{cat}/K_M value for the nucleophile mutant, D224A, was 10000-fold lower than that of the wild type. As was done with BT2970fuc, the residue in Tmfuc that aligns with the residue assigned as the acid/base in Hfuc (E281 in Tmfuc and E289 in Hfuc) was mutated to alanine to probe a possible role. The E281A mutant of Tmfuc was again found to hydrolyze pNPαFuc just as efficiently as does wild-type Tmfuc, suggesting also that this residue does not play a role in catalysis or binding in Tmfuc either (Table 3) and calling into question local alignments or suggesting further restructuring of active site loops in Hfuc upon substrate binding.

Kinetic Characterization of Subfamily B Enzymes BT4136fuc and BT1625fuc and Their Mutants. A total of six aspartate and glutamate residues were chosen for mutation: E249, D286, E294, E298, E305, and E307 (the numbering is the same for BT4136 and BT1625). Residues E249 and E305 were chosen because they aligned with two preferred candidate acid/base residues. E305 is fully conserved and aligns with the acid/base residue found for Hfuc, while E249 is the residue that undergoes a large change in position as a consequence of the substrate-induced conformational change and has been assigned as a probable acid/base on the basis of the X-ray structure of the *Bifidobacter* enzyme.[11] D286 and D307 were chosen because they are also reasonably conserved in sequence alignments and thus remained possible candidates for the role of the acid/base. Residues E294 and E298 were chosen, despite imperfect alignments, because they are located in a region with a string of aromatic residues (FWY) that could be involved in hydrophobic stacking interactions with the

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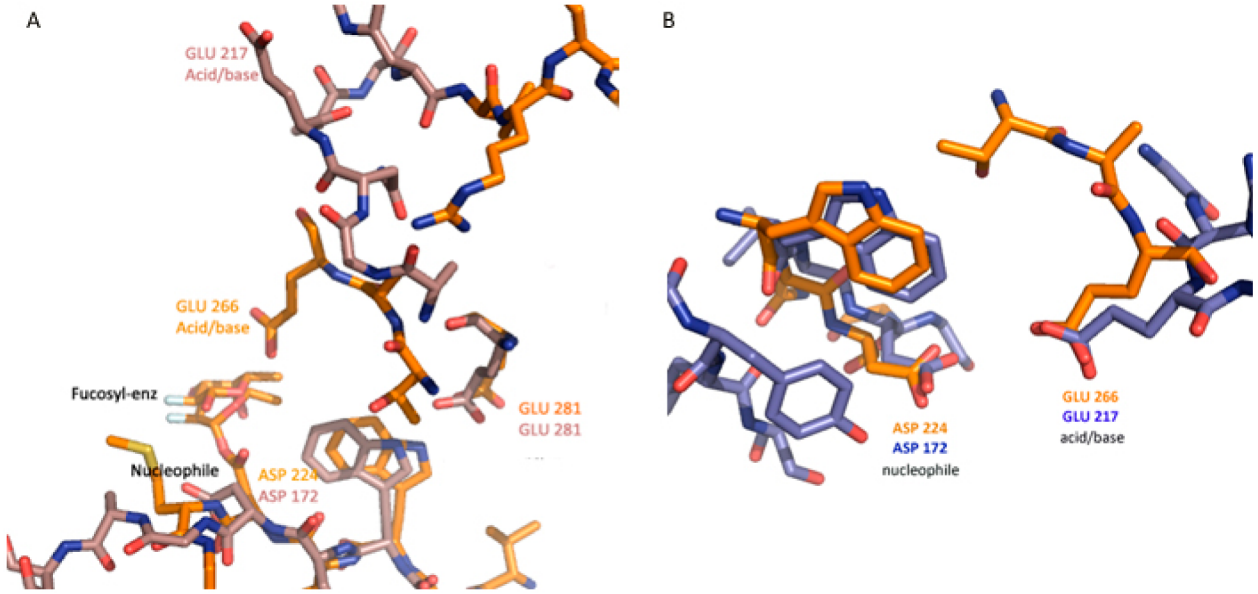

Figure 3. Comparison of structures of Tmfuc (subfamily A) and BiAfcB (subfamily B) in free and substrate-bound states. Tmfuc is colored orange, BiAfcB (free, A) purple, and BiAfcB (bound, B) blue.

substrate. Of these, unfortunately E294A could not be expressed.

Kinetic parameters for hydrolysis of pNPαFuc and αFucF by the wild type and alanine mutants of BT4136fuc and BT1625fuc are reported in Table 3. As was seen with BT2970, comparison of the k_{cat} values for the hydrolysis of pNPαFuc and αFucF by BT4136fuc and BT1625fuc suggests that deglycosylation is likely rate-limiting for the wild-type enzymes. Likewise, mutation of the nucleophile residue, D204, to an alanine gave rise to a completely inactive enzyme, consistent with its role. Mutation of the best candidate for the acid/base residue, E249, to alanine gave rise to an enzyme with a k_{cat}/K_M value for hydrolysis of pNPαFuc that is 3200-fold (for BT4136) and 10000-fold (for BT1625) lower than that observed with the wild type, consistent with the loss of acid/base assistance by mutants. By contrast, the k_{cat}/K_M value for the hydrolysis of αFucF by the E249A mutant was only 15−17-fold lower than that of the wild type. Such a difference in the ratio of k_{cat}/K_M values (wild type to acid/base) between the two substrates is expected as explained previously and provides support for E249 being the acid/base residue for BT4136 and BT1625, as also does the 36-fold lower K_M for hydrolysis of αFucF by E249A than by WT (Table 4). The deglycosylation step is likely rate-limiting for these E294A acid/base mutants because very similar k_{cat} values were observed for both substrates, these being 650−1000-fold lower than wild-type values. This is a reduction in rate similar to that seen with the acid/base catalyst of the subfamily A enzymes and indeed to that seen for many retaining glycosidases in the absence of general base catalysis.[19,20]

Mutation of E305 (the residue that aligns with the acid/base residue for Hfuc) to alanine did not significantly affect the activity or binding of the substrate, just as seen for the equivalent residue in subfamily A; therefore, it is not involved in acid/base catalysis in this enzyme. Likewise, mutations of D286 and E298 also were largely without effect; thus, it is unlikely that they are directly involved in catalysis or binding. An approximate 10-fold decrease in activity and a slight increase in K_M were observed upon mutation of D307 to an alanine residue, though the magnitude of this decrease suggests that this residue is not critical to catalysis.

Small Molecule Rescue of Acid/Base (and nucleophile) Mutants. Small molecule rescue was performed to further validate the roles of the putative acid/base residues, E288 (for BT2970, subfamily A) and E249 (for BT4136 and BT1625, subfamily B), in catalysis. Rescue of the nucleophile mutants, while not critical to this study, is summarized in Table 4 and showed that activity could be rescued as expected. More importantly, 3-, 4.5-, and 4.5-fold increases in k_{cat} for the hydrolysis of pNPαfuc by the E288A, BT4136fuc E249A, and BT1625 E249A mutants, respectively, were seen upon inclusion of 200 mM sodium azide in reaction mixtures (Table 4). Equivalent levels of rescue have been seen for a range of α-glycosidases, consistent with deglycosylation being rate-limiting in its absence.[9,30] Azide "rescue" was not seen for any of the other mutants, and no increase (or decrease) in wild-type activity was observed in the presence of azide. The presence of azide rescue provides strong support for the assignment of these proposed acid/base residues. TLC analysis of the product(s) obtained from the hydrolysis of pNPαfuc by the acid/base mutants (E249A of BT4136 and E288A of BT2970) reveals a new product (R_f = 0.71) formed in the presence of azide. This runs at a different R_f than L-fucose ($R_f \sim 0.46$) or pNPαFuc (R_f = 0.81). After purification, mass spectral analysis confirmed that a product that has a molecular formula consistent with fucosyl azide was formed: m/z calcd for $C_6H_{11}N_3O_4Na^+$ 212.0593, found 212.0596.

CONCLUSIONS

Kinetic analysis of selected mutants within the two subfamilies using substrates with differing leaving group abilities, along with azide rescue experiments, confirmed the proposed acid/base residues in each. Thus, assignment of E288 as the acid/base residue in BT2970 (subfamily A) is supported by kinetic data, azide rescue, and the observation of the trapped glycosyl−enzyme intermediate obtained upon the reaction of αFucF with the E288Q mutant by Lammerts van Bueren et al.[27] Kinetic data and azide rescue also provide support for the assignment

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of E249 as the acid/base residue for BT4136 and BT1625 (subfamily B). Indeed, the acid/base residues of these models overlap with that of BiAfcB, in which the conformational change was experimentally demonstrated by Sakurama et al., further supporting their assignment as the acid/base.[11] Figure 3A shows the spatial relationships of these residues for unliganded structures of representatives of subfamilies A and B, while Figure 3B shows the structure of the subfamily B *Bifidobacter* enzyme BiAfcB with a ligand bound.

Kinetic data and azide rescue data obtained by Liu et al. provide support for E289 as the acid/base residue for Hfuc (subfamily A).[14] This residue does not align with acid/base residues of Tmfuc or BiAfcB, suggesting that a different conformational change would need to occur in this case. It is noteworthy that an unstructured region was found in the Tmfuc structure close to E281, possibly consistent with the presence of a mobile region in the human enzyme. It is also of interest that the eukaryotic members of subfamily A form a subbranch, within which it is possible that this alternative mobile region may be present. As no crystal structure is available for the human GH29 fucosidase, or for any eukaryotic GH29 enzymes, predictions regarding the specific conformational change that would need to occur are not currently substantiated. Such a structure is sorely needed to fill the final remaining gap in our understanding of this important enzyme class.

■ ASSOCIATED CONTENT

Supporting Information

A table showing forward and reverse primers used for generating mutants using the QuickChange method. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

αFucF, α-L-fucopyranosyl fluoride; BT, *B. thetaiotaomicron*; DFJ, deoxyfuconojirimycin; Hfuc, human α-L-fucosidase; pNPαFuc, *p*-nitrophenyl α-L-fucoside; R_f, retention factor; Tmfuc, α-L-fucosidase from *T. maritima*.

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