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# Identification of the Catalytic Nucleophile of the Family 29 $\alpha$ -L-Fucosidase from *Thermotoga maritima* through Trapping of a Covalent Glycosyl-Enzyme Intermediate and Mutagenesis\*

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**Fucose-containing glycoconjugates are key antigenic determinants in many biological processes. A change in expression levels of the enzymes responsible for tailoring these glycoconjugates has been associated with many pathological conditions and it is therefore surprising that little information is known regarding the mechanism of action of these important catabolic enzymes. *Thermotoga maritima*, a thermophilic bacterium, produces a wide range of carbohydrate-processing enzymes including a 52-kDa  $\alpha$ -L-fucosidase that has 38% sequence identity and 56% similarity to human fucosidases. The catalytic nucleophile of this enzyme was identified to be Asp-224 within the peptide sequence <sup>222</sup>WNDMGWPE-KGKEDL<sup>235</sup> using the mechanism-based covalent inactivator 2-deoxy-2-fluoro- $\alpha$ -L-fucosyl fluoride. The 10<sup>4</sup>-fold lower activity ( $k_{cat}/K_m$ ) of the site-directed mutant D224A, and the subsequent rescue of activity upon addition of exogenous nucleophiles, conclusively confirms this assignment. This article presents the first direct identification of the catalytic nucleophile of an  $\alpha$ -L-fucosidase, a key step in the understanding of these important enzymes.**

$\alpha$ -L-Fucosidases (EC 3.2.1.51) are exoglycosidases unique to family 29 in the sequence-based classification of glycoside hydrolases (1).<sup>1</sup> They are responsible for the removal of L-fucosyl residues from the non-reducing end of glycoconjugates, the most common linkages being  $\alpha$ 1–2 to galactose and  $\alpha$ 1–3,  $\alpha$ 1–4, and  $\alpha$ 1–6 to *N*-acetylglucosamine residues. Inactivity of this enzyme leads to the accumulation of fucose-containing glycolipids and glycoproteins in various tissues and results in the clinical condition fucosidosis (3). In humans this autosomal recessive lysosomal storage disease can arise from many different mutations to the *FUCA1* gene and, while rare, is a terminal degenerative condition (4). Fucose-containing oligosaccharides are involved in many key cellular interactions such as inflammatory response (5–7) and antigenic determination (8).

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<sup>1</sup> P. M. Coutinho and B. Henrissat, Carbohydrate-Active Enzymes server: afmb.cnrs-mrs.fr/~cazy/CAZY/index.

Furthermore, changes in fucosylation levels have been observed in many carcinomas (9, 10). Although many  $\alpha$ -L-fucosidases have been identified, their detailed catalytic mechanism and the identities of key active site residues have not yet been elucidated. A complete understanding of the mechanism of action of this class of enzymes is required to enable therapeutic intervention.

Recent work has demonstrated that  $\alpha$ -L-fucosidases are retaining enzymes; the hydrolyzed hemiacetal product bears the same stereochemical configuration as the original glycoside (11, 12). In the vast majority of these cases this stereochemical outcome arises from a double displacement mechanism within the enzyme active site. This mechanism requires the action of two critical amino acid residues with specific roles for catalysis: one functioning as a general acid/base, the other as a nucleophile. In this class of enzymes these residues are usually found to be carboxylic acids. Glycoside hydrolysis requires the action of the general acid catalyst to assist in the departure of the aglycone oxygen. Simultaneously the other carboxylate residue acts as a nucleophile, attacking the anomeric center of the glycone, generating a covalent glycosyl-enzyme intermediate. Upon diffusion of the aglycone from the active site, this glycosyl-enzyme is subsequently hydrolyzed with general base assistance from the acid/base residue. The hemiacetal thus released retains the same anomeric orientation as the original glycoside because an overall double inversion of configuration has taken place. The identification of these key residues is of fundamental importance to the understanding of the detailed molecular mechanism through which these enzymes operate.

## EXPERIMENTAL PROCEDURES

**General Procedures and Synthesis**—All buffer chemicals and other reagents were obtained from the Sigma unless otherwise noted. 2-Deoxy-2-fluoro- $\alpha$ -L-fucosyl fluoride was synthesized by known methodology (13).

**Cloning, Purification, and Mutagenesis**—The coding sequence of the *Thermotoga maritima* (TM) gene TM0306 was amplified by PCR from genomic DNA (kindly provided by Dr. Wolfgang Liebl) and subcloned into the pDEST17 plasmid (Gateway, Invitrogen) introducing a 15-residue linker and a His<sub>6</sub> tag at the N terminus. Protein expression was carried out in *Escherichia coli* BL21(DE3) pLysS strain and cells were lysed by lysozyme treatment and subsequent freeze-thawing. Protein was purified by Ni<sup>2+</sup> affinity and size exclusion chromatographies and sample purity was assessed by SDS-PAGE.

The D224A mutant was generated using the QuikChange<sup>TM</sup> mutagenesis kit (Stratagene), verified by DNA sequencing, but the protein expressed in the conditions described above was insoluble. The coding sequence was therefore subcloned into a derivative of the expression vector pKM596 (14) in which a His<sub>6</sub> tag was introduced upstream of the coding sequence of the maltose-binding protein, allowing the expression

of the mutant as a His<sub>6</sub>-MBP<sup>2</sup> fusion protein. Expression and purification were carried out as for the native enzyme. The wild type protein was also expressed and purified as a His<sub>6</sub>-MBP fusion protein for comparison. A detailed description of the cloning, expression, purification, and mutagenesis steps of TM  $\alpha$ -L-fucosidase will be described elsewhere.<sup>3</sup>

**Labeling and Proteolysis**—Labeling of *T. maritima*  $\alpha$ -L-fucosidase was accomplished by incubating the His<sub>6</sub>-wild type enzyme (2.43 mg ml<sup>-1</sup> final concentration) and 2-deoxy-2-fluoro- $\alpha$ -L-fucosyl fluoride (2.5 mM final concentration) in pH 5.0, 50 mM citrate/phosphate buffer (80  $\mu$ l total volume) for 1 h at room temperature. After this time digestion buffer (120  $\mu$ l), pH 2.0, containing pepsin (0.3 mg ml<sup>-1</sup>) was added. Proteolytic digestion was performed for 1 h and the sample was frozen prior to mass spectrometric analysis. A sample of unlabeled enzyme for comparison was also prepared in the same manner.

**Electrospray Mass Spectrometric Analysis of the Proteolytic Digest**—Mass spectra were recorded on a PE-Sciex API 300 triple-quadrupole mass spectrometer and a PE-Sciex API QSTAR pulsar i (Sciex, Thornhill, Ontario, Canada) equipped with an ionspray ion source. Peptides were separated by reverse-phase HPLC on a LC Packing Ultimate Micro HPLC system (Dionex, Sunnyvale, CA) directly interfaced with the mass spectrometer. In each of the MS experiments the proteolytic digest was loaded onto a C-18 column (LC Packing, 100- $\text{\AA}$  pepMap, 1  $\times$  150 mm) equilibrated with solvent A (0.05% trifluoroacetic acid, 2% acetonitrile in water). Elution of the peptides was accomplished using a gradient (0–60%) of solvent B over 60 min followed by 85% solvent B over 20 min (solvent B: 0.045% trifluoroacetic acid, 80% acetonitrile in water). Solvents were pumped at a constant flow rate of 50  $\mu$ l/min. Spectra were recorded in the single quadrupole scan mode or the tandem MS product-ion scan mode. In the single-quadrupole mode the quadrupole mass analyzer was scanned over a mass to charge ratio (*m/z*) range of 300–2200 Da with a step size of 0.5 Da and a dwell time of 1.5 ms per step. The ion source voltage was set at 5.5 kV, and the orifice energy was 45 V. In the tandem MS daughter-ion scan mode, the spectra were obtained in a separate experiment by selectively introducing the labeled (*m/z* = 927.5) or unlabeled (*m/z* = 853) parent ion from the first quadrupole (Q1) into the collision cell (Q2) and observing the product ions in the third quadrupole (Q3). The scan range of Q3 was 50–1100; the step size was 0.5; the dwell time was 1 ms; ion source voltage was 5 kV; orifice energy was 45 V; Q0 = -10; and IQ2 = -48.

**Metal Ion Dependence**—Aliquots (100  $\mu$ l) of His<sub>6</sub>-wild type and MBP-wild type  $\alpha$ -L-fucosidase were placed into a mini dialysis unit (Pierce) with a molecular weight cut off of 10,000. These units were then dialyzed against a buffer of pH 7.5, 15 mM HEPES containing 10 mM EDTA for 2 h to remove any contaminating divalent metal ions. The dialysis buffer solution was then changed for a fresh solution and dialysis continued for a further 12 h. The dialysis units were then dialyzed against a fresh, pH 7.5, 5 mM HEPES buffer for 2 h to remove residual EDTA. Again the buffer was changed for a fresh solution and dialysis continued for a further 12 h. Enzyme activity of these two proteins was then assayed in the manner described below both in the presence and absence of divalent metal cations.

**Enzyme Kinetics**—All kinetic studies were performed in 50 mM buffer containing 145 mM sodium chloride and 0.1% bovine serum albumin. For the pH range 3.0–6.5 a sodium citrate/sodium phosphate buffer was used. Sodium phosphate buffer was used between pH 6.5 and 8.0. Kinetic measurements using the *p*NP-fucoside substrate and the His<sub>6</sub>-wild type enzyme were performed at 60 °C, the enzyme showing no loss of activity after 24 h incubation at this temperature. Reactions involving the MBP were performed at the reduced temperature of 37 °C. A total reaction volume of 800  $\mu$ l was used in these assays. At pH 5.5 and above a continuous spectrophotometric assay was used to monitor the release of the *p*-nitrophenolate anion at 400 nm. For measurements at acidic pH the *p*-nitrophenol product was monitored at 360 nm. Extinction coefficients were determined for each pH point at 60 °C by the method of Kempton and Withers (15); pH 3.55,  $\epsilon(360 \text{ nm}) = 1.66 \text{ mM}^{-1} \text{ cm}^{-1}$ ; pH 4.02,  $\epsilon(360 \text{ nm}) = 1.70 \text{ mM}^{-1} \text{ cm}^{-1}$ ; pH 4.45,  $\epsilon(360 \text{ nm}) = 1.74 \text{ mM}^{-1} \text{ cm}^{-1}$ ; pH 5.0,  $\epsilon(360 \text{ nm}) = 1.81 \text{ mM}^{-1} \text{ cm}^{-1}$ ; pH 5.51,  $\epsilon(400 \text{ nm}) = 2.13 \text{ mM}^{-1} \text{ cm}^{-1}$ ; pH 6.02,  $\epsilon(400 \text{ nm}) = 3.66 \text{ mM}^{-1} \text{ cm}^{-1}$ ; pH 6.52,  $\epsilon(400 \text{ nm}) = 7.38 \text{ mM}^{-1} \text{ cm}^{-1}$ ; pH 6.99,  $\epsilon(400 \text{ nm}) = 11.39 \text{ mM}^{-1} \text{ cm}^{-1}$ ; pH 7.45,  $\epsilon(400 \text{ nm}) = 14.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ; pH 8.03,  $\epsilon(400 \text{ nm}) = 17.26 \text{ mM}^{-1} \text{ cm}^{-1}$ .

cm<sup>-1</sup>. Spectroscopic measurements were performed on temperature-stabilized Cary AV-300 and Cary AV-4000 spectrophotometers. The hydrolysis of the  $\alpha$ -L-fucosyl fluoride substrates was monitored using a 96–09 Ionplus Orion fluoride ion selective electrode to detect the release of fluoride. This instrument was coupled to a PC and the data collected using the Logger Pro<sup>TM</sup> program (Vernier Software Inc.). These assays were performed at 25 °C to minimize substrate hydrolysis and were conducted in glass vials with a total assay volume of 700  $\mu$ l. For each Michaelis-Menten curve generated the initial rates of 6–8 substrate concentrations were used. Wherever possible the substrate concentration was varied from 0.2 to 5 times  $K_m$ . The kinetic parameters  $k_{\text{cat}}$  and  $K_m$  were calculated by fitting of the initial rate data to the Michaelis-Menten equation using Grafit version 4.0 (16). In all cases the observed rate was corrected for background substrate hydrolysis. Similar methodology was used for the analysis of methanol as a competitive nucleophile. TLC analysis of products was performed using aluminum-backed Merck Silica Gel 60 F<sub>254</sub> plates (chloroform:methanol:water, 20:6:1).

**pH Dependence**—Because of the low  $K_m$  of *p*NP-fucoside, the substrate depletion method for determination of the apparent second-order rate constant  $k_{\text{cat}}/K_m$  could not be applied. The pH dependence of the His<sub>6</sub>-wild type enzyme was therefore determined by performing standard Michaelis-Menten kinetic analysis at increments of 0.5 pH units.

**Chemical Rescue of Alanine Mutant**—Assays with azide were performed at pH 6.0 in 50 mM citrate/phosphate buffer. This perturbation from the pH optimum, pH 4.5–5.0, was required because of the relatively high  $\text{p}K_a$  of hydrazoic acid ( $\text{p}K_a$  4.8). An additional benefit of this pH was the increased extinction coefficient of the *p*-nitrophenolate anion. Sodium azide (4 M, buffered to pH 6.0) was added at varying concentrations and the rate of product release monitored. Assays with formate as rescue agent were performed at pH 5.0 in a similar fashion with substrate concentrations in the range of 400–600  $\mu$ M. Assays of the D224A MBP fusion protein with *p*NP-fucoside were conducted at a temperature of 37 °C because of the lower thermal stability of the MBP construct. Reaction times were increased to 4–12 h to compensate for the very slow rate of reaction; the reaction was observed to be linear across this time range, indicating no loss of enzyme activity.

## RESULTS AND DISCUSSION

**Metal Ion Dependence**—Because metal ion affinity chromatography was used in the purification of the His<sub>6</sub>-wild type enzyme, an analysis of any possible metal ion dependence of the enzyme was carried out. No loss of activity of the His<sub>6</sub>-wild type or the MBP-wild type enzymes was observed upon extensive dialysis against EDTA. Inclusion of 10 mM EDTA in the reaction mixture also did not affect the rate of enzyme-catalyzed hydrolysis. Addition of various divalent metal cations (calcium, cobalt, magnesium, manganese, nickel, and zinc) at 10 mM concentrations did not increase enzymatic rates; only a small decrease being observed in some cases. A small positive salt effect was noticed at high concentrations of sodium chloride, therefore subsequent assays were performed in the presence of 145 mM sodium chloride.

**Kinetic Analysis and pH Dependence**—Kinetic parameters for the cleavage of *p*NP-fucoside by the His<sub>6</sub>-wild type enzyme were determined and a representative plot is shown in Fig. 1. As can be seen, substrate inhibition was observed at high *p*NP-fucoside concentrations. Fitting these data to a substrate inhibition model in Grafit 4.0, in which inhibition arises from the binding of a second substrate molecule to form an ES<sub>2</sub> complex that is catalytically inactive (17), yields values of  $k_{\text{cat}} = 9.4 \text{ s}^{-1}$ ,  $K_m = 35 \text{ }\mu\text{M}$ , and  $K_i = 640 \text{ }\mu\text{M}$  at pH 5.0. The  $k_{\text{cat}}$  and  $K_m$  values were in close agreement to those obtained when only low substrate concentrations (0–100  $\mu$ M) were analyzed by best fit to the Michaelis-Menten equation;  $k_{\text{cat}} = 9.1 \text{ s}^{-1}$ ,  $K_m = 32 \text{ }\mu\text{M}$ . In light of this observation the maximum concentration of *p*NP-fucoside employed in further experiments was 100  $\mu$ M. Values of  $k_{\text{cat}}$  and  $K_m$  were determined at 60 °C for the hydrolysis of *p*NP-fucoside by  $\alpha$ -L-fucosidase-His<sub>6</sub> as a function of pH. The enzyme was found to be unstable at pH 3.0 with rapid inactivation occurring. This inactivation was assigned to enzyme denaturation because activity could not be recovered

<sup>2</sup> The abbreviations used are: MBP, maltose-binding protein; *a* → *a*, axial → axial; *e* → *e*, equatorial → equatorial; *p*NP, 4-nitrophenol; HPLC, high performance liquid chromatography.

<sup>3</sup> G. Sulzenbacher, C. Bignon, T. Nishimura, T. C. Tarling, S. G. Withers, B. Henrissat, and Y. Bourne, manuscript in preparation.

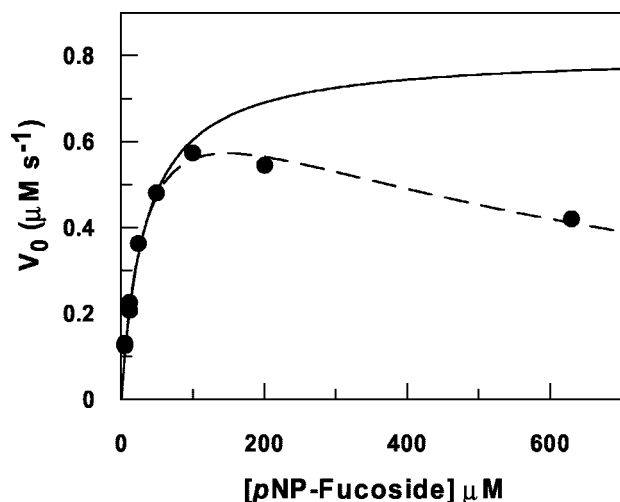


FIG. 1. Typical kinetic data for the enzyme catalyzed hydrolysis of *p*NP-fucoside by the *T. maritima*  $\alpha$ -L-fucosidase. The solid line represents the best fit to the Michaelis-Menten equation where only data from substrate concentrations of 0–100  $\mu$ M are included. This yields kinetic parameters of  $k_{\text{cat}} = 9.1 \pm 0.5 \text{ s}^{-1}$  and  $K_m = 32 \pm 3 \mu\text{M}$ . The dashed line shows the best fit of all data to a substrate inhibition model in Grafit 4.0 and provides values of  $k_{\text{cat}} = 9.4 \pm 0.4 \text{ s}^{-1}$ ,  $K_m = 35 \pm 3 \mu\text{M}$ , and  $K_i = 640 \pm 80 \mu\text{M}$ . The reactions were performed at 60 °C using concentrations of the *p*NP-fucoside substrate from 5 to 620  $\mu\text{M}$ .

upon restoring the pH to 6.0. The pH dependence of  $k_{\text{cat}}/K_m$  shows a typical bell-shaped curve expected from the presence of two ionizable carboxylic acid residues in the enzyme active site with a pH optimum of 5.0 (Fig. 2). Fitting the data to a double titration curve to yield  $\text{p}K_a$  values of these two residues in the free enzyme gave estimates of 3.8 and 6.1 ( $\pm 0.1$ ), presumably the catalytic nucleophile and acid/base catalyst, respectively. The pH dependence of  $k_{\text{cat}}$ , which represents the ionization of the two key catalytic residues during the rate-determining step of catalysis, is also seen to be a bell-shaped curve with an optimum at pH 4.5 (Fig. 2). The  $\text{p}K_a$  values in this case are similar, being 3.4 and 5.7 ( $\pm 0.1$ ), although the acidic limb of this profile is not well defined because of the limited stability of the enzyme at low pH.

$\alpha$ -L-Fucosyl fluoride was also investigated as a substrate, these assays being performed at 25 °C because of the significant spontaneous hydrolysis of the fucosyl fluoride at elevated temperatures (Table I). The catalytic activity ( $k_{\text{cat}}$ ) of the His<sub>6</sub>-wild type enzyme with this substrate was similar to that observed for *p*NP-fucoside, however, no inhibition was observed at higher substrate concentrations. The higher  $K_m$  observed for fucosyl fluoride is most reasonably rationalized by the absence of an aglycone able to contribute binding interactions in the +1 subsite. That a significantly increased rate was not observed upon replacement of the aglycone with a considerably superior leaving group is highly indicative of a deglycosylation rate-limiting step for both of these substrates ( $\text{p}K_a$  *p*NP = 7.2,  $\text{p}K_a$  hydrogen fluoride = 3.2). To demonstrate that deglycosylation is in fact the rate-determining step for these substrates a nucleophilic competition experiment was performed. The addition of an uncharged exogenous nucleophile to the reaction mixture would not be expected to have any effect upon the rate of the first step of enzyme catalysis (glycosylation). Therefore, were this to be the rate-determining step, no increase in steady state rate would be expected. However, the rate of deglycosylation may be increased because of the faster cleavage of the glycosyl-enzyme intermediate with the exogenous nucleophile than with water. Addition of various concentrations of methanol to the reaction mixture caused a linear increase in rate with

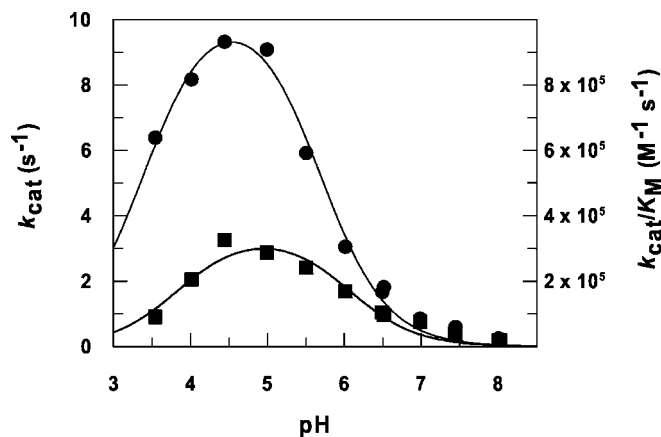


FIG. 2. pH dependence of  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_m$  of wild type *T. maritima*  $\alpha$ -L-fucosidase. The lines represent the best fit of the data to the double titration model in Grafit 4.0; (●)  $k_{\text{cat}}$ ; (■)  $k_{\text{cat}}/K_m$ . All reactions were performed at 60 °C with *p*NP-fucoside as substrate. The enzyme was too unstable at pH values of less than 3.5 for any kinetic parameters to be determined.

increasing methanol concentration for both *p*NP-fucoside and fucosyl fluoride (Fig. 3). TLC analysis of the products formed in this reaction showed a new product ( $R_F = 0.60$ ) with an intermediate mobility between *p*NP-fucoside ( $R_F = 0.76$ ) and fucose ( $R_F = 0.26$ ). This new product was observed to co-run with an authentic sample of methyl- $\alpha$ -L-fucopyranoside, confirming that the expected product is indeed formed. Formation of this new product was not observed in control reactions performed in the absence of either methanol or enzyme. Deglycosylation is therefore at least partially rate-limiting for both of these substrates.

*Approaches to Mechanism-based Inhibition*— $\alpha$ -L-Fucosidases can adopt two low energy chair conformations,  ${}^4C_1$  and  ${}^1C_4$ , the  ${}^1C_4$  conformation being predominantly preferred in solution (Fig. 4). This latter conformation, with an axial oriented aglycone, may undergo enzymatic cleavage in a fashion similar to that of  $\alpha$ -D-retaining enzymes; such a reaction being described as  $a \rightarrow a$  (18). Conversely the  ${}^4C_1$  conformer might be processed by an  $e \rightarrow e$  mechanism analogous to  $\beta$ -D-glycosidases.

The importance of this  $a \rightarrow a$  or  $e \rightarrow e$  classification is not merely represented by the nomenclature imposed but rather by the different approaches that must be employed to successfully label the catalytic nucleophile. The primary method for the identification of the catalytic nucleophile in retaining glycosidases has been through the use of substrate analogs that form a covalent glycosyl-enzyme intermediate at a rate that far exceeds that of the subsequent hydrolysis step. The consequence of this situation is the accumulation of a glycosyl-enzyme intermediate, and in many cases complete inactivation of enzyme-catalyzed hydrolysis. Proteolysis can then be used to create small peptides for analysis by liquid chromatography coupled to a tandem mass spectrometer and the location of the modified carboxylate residue identified. In the case of  $\beta$ -retaining glycosidases, activated 2-deoxy-2-fluoroglycosides have proven very successful (19–22). This approach has been found to be less successful with  $\alpha$ -retaining glycosidases where these 2-fluorosugars are generally observed to be slow substrates for which the glycosylation step is rate-limiting (23, 24). In this situation no glycosyl-enzyme intermediate accumulates. To address this problem, two other classes of mechanism-based inactivators have been developed. These are the 2,2-difluoroglycosides (25), which incorporate very activated leaving groups, and the 5-fluoroglycosyl fluorides (26), both of which have been used successfully to label the catalytic nucleophiles of  $\alpha$ -retaining glycosidases (27).

TABLE I  
Kinetic parameters for hydrolysis of fucoside substrates by *T. maritima*  $\alpha$ -L-fucosidase

Michaelis-Menten parameters for the hydrolysis of *p*NP-fucoside and fucosyl fluoride substrates by the wild type and D224A mutant of the *T. maritima*  $\alpha$ -L-fucosidase.

Enzyme	Substrate	$k_{\text{cat}}$ $\text{s}^{-1}$	$K_M$ $\mu\text{M}$	$k_{\text{cat}}/K_M$ $\text{M}^{-1} \text{s}^{-1}$
Wild type	<i>p</i> NP-fucoside <sup>a</sup>	$5.4 \pm 0.2$	$34 \pm 2$	$1.6 \pm 0.1 \times 10^5$
MBP-wild type	<i>p</i> NP-fucoside <sup>a</sup>	$14.3 \pm 0.3$	$50 \pm 3$	$2.8 \pm 0.2 \times 10^5$
MBP-D224A	<i>p</i> NP-fucoside <sup>a</sup>	ND <sup>b</sup>	ND	$28 \pm 1^c$
Wild type	Fucosyl fluoride <sup>d</sup>	$2.9 \pm 0.1$	$230 \pm 20$	$1.3 \pm 0.1 \times 10^4$
MBP-wild type	Fucosyl fluoride <sup>d</sup>	$7.6 \pm 0.2$	$150 \pm 10$	$5.0 \pm 0.4 \times 10^4$
D224A	Fucosyl fluoride <sup>d</sup>	$<0.01^e$	ND	ND

<sup>a</sup> Measured at pH 5.0, 37 °C.

<sup>b</sup> ND, not determined.

<sup>c</sup> No saturation observed.

<sup>d</sup> Measured at pH 5.0, 25 °C.

<sup>e</sup> At 500  $\mu\text{M}$  fucosyl fluoride.

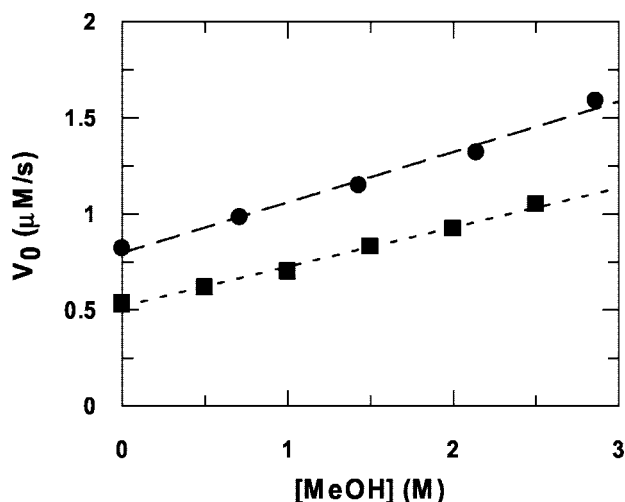


FIG. 3. The effect of methanol on the rate of substrate cleavage by the *T. maritima*  $\alpha$ -L-fucosidase. The linear increase in enzyme-catalyzed cleavage is shown for fucosyl fluoride (●) and *p*NP-fucoside (■). Reactions with *p*NP-fucoside were performed at 100  $\mu\text{M}$  substrate, 60 °C, pH 5.0. Reactions with fucosyl fluoride were performed at 450  $\mu\text{M}$  substrate, 25 °C, pH 5.0.

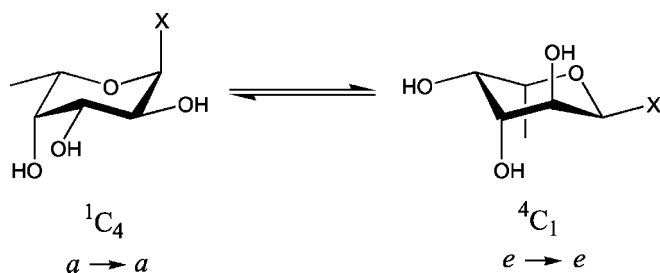


FIG. 4. Possible chair conformations of  $\alpha$ -L-fucopyranosides and the implication for the catalytic mechanism. The two possible chair conformations,  ${}^1C_4$  and  ${}^4C_1$ , for  $\alpha$ -L-fucopyranosides are shown, X = aglycone. The  ${}^1C_4$  conformer could follow an enzymatic reaction mechanism analogous to the axial – axial ( $a \rightarrow a$ ) transformation observed with retaining  $\alpha$ -D-glycosidases. The  ${}^4C_1$  conformer on the other hand more closely resembles the  $\beta$ -D-glycosides that are processed by an equatorial – equatorial ( $e \rightarrow e$ ) mechanism.

The classification of both  $\alpha$ -L-iduronidases and  $\beta$ -D-xylosidases to the same glycoside hydrolase family 39 attests to the occasional commonality between  $\alpha$ -L- and  $\beta$ -D-glycosides. Moreover, recent work within this laboratory has conclusively demonstrated that  $\alpha$ -L-iduronidases and  $\beta$ -D-xylosidases react through similar glycosyl-enzyme intermediates (28). In that study the catalytic nucleophile of the human  $\alpha$ -L-iduronidase was labeled using both a 5-fluoroglycosyl fluoride and a 2-deoxy-2-fluoroglycosyl fluoride analog of iduronic acid. Because

$\alpha$ -L-fucosidases are alone in family 29, with no other glycosidases assigned to that family, there is some ambiguity regarding the nature of the mechanism ( $a \rightarrow a$  versus  $e \rightarrow e$ ). Based on the success of the 2-deoxy-2-fluorosugar approach with the human  $\alpha$ -L-iduronidase (28), we elected to first synthesize and test the more readily accessible 2-deoxy-2-fluorofucosyl fluoride as a probe to identify the catalytic nucleophile of the *T. maritima*  $\alpha$ -L-fucosidase. The synthesis of this mechanistic probe was readily achieved following known methodology (13).

**Reaction of  $\alpha$ -L-Fucosidase with 2-Deoxy-2-fluoro- $\alpha$ -L-fucosyl Fluoride**—Incubation of the enzyme with 2-deoxy-2-fluoro- $\alpha$ -L-fucosyl fluoride did not lead to time-dependent inhibition of activity. To determine whether this compound was instead behaving as a substrate, or merely not binding in the enzyme active site, kinetic analysis of its cleavage using a fluoride ion-selective electrode was undertaken. Monitoring the rate of fluoride release showed that 2-deoxy-2-fluoro- $\alpha$ -L-fucosyl fluoride was indeed a slow Michaelian substrate with  $k_{\text{cat}} = 0.047 \pm 0.003 \text{ s}^{-1}$  and  $K_m = 2.1 \pm 0.2 \text{ mM}$ . This  $k_{\text{cat}}$  value is very similar to those previously published for fluorosugar substrates with other  $\alpha$ -glycosidases;  $0.022 \text{ s}^{-1}$  for 5-fluoro- $\beta$ -L-gulosyl fluoride with the family 38  $\alpha$ -mannosidase from bovine kidney (29) and  $0.055 \text{ s}^{-1}$  observed with 5-fluoro- $\alpha$ -D-glucosyl fluoride and the family 31  $\alpha$ -glucosidase from *Aspergillus niger* (30). The substantially increased  $K_m$  of 2.1 mM over fucosyl fluoride ( $K_m = 232 \mu\text{M}$ ) is likely because of the loss of key binding interactions with the 2-hydroxyl group. The 2-hydroxyl group is apparently involved in key interactions necessary for both binding and catalysis because the purely electronic difference of this substitution ( $\Delta\sigma_1 = 0.25$  for OH  $\rightarrow$  F) is unlikely to be solely responsible for the 2300-fold difference in catalytic efficiency (31). This is consistent with previous observations of the key role of the 2-hydroxyl in catalysis (32).

**Identification of the Active Site Nucleophile**—No inhibition of enzyme activity, time-dependent or competitive, was observed in the presence of 2-deoxy-2-fluoro- $\alpha$ -L-fucosyl fluoride. Whereas this might suggest that no intermediate accumulates, it is quite possible that any accumulated intermediate turns over rapidly via transglycosylation to *p*NP-fucoside during the activity assay. This situation has previously been observed with other glycosidases inactivated with fluorosugars (22). However, a rate-limiting deglycosylation step for 2-deoxy-2-fluoro- $\alpha$ -L-fucosyl fluoride, which would result in accumulation of the intermediate, seemed quite probable given that such is the case for *p*NP-fucoside and presumably also  $\alpha$ -L-fucosyl fluoride, given the similarities in  $k_{\text{cat}}$  values. Unfortunately because the intact protein was not amenable to electrospray mass spectrometric analysis we could not look for such accumulation directly. It was therefore necessary to analyze the peptides formed after proteolysis. Following incubation of the enzyme

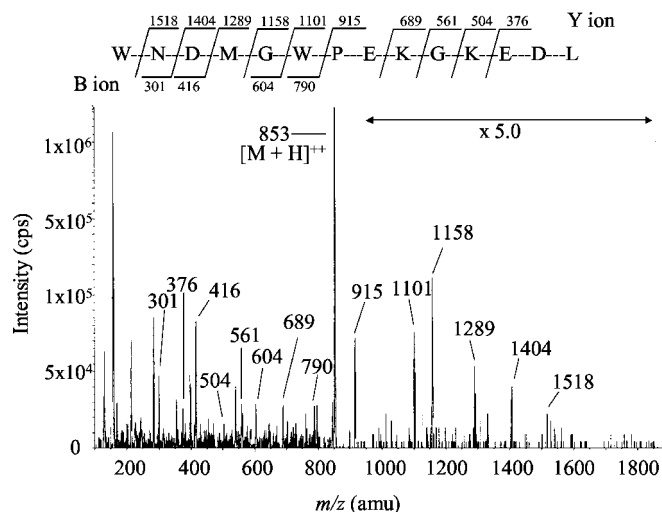


FIG. 5. Electrospray tandem MS daughter ion spectrum of the purified unlabeled peptide. The peptide that bears the fucosyl label was identified by comparative mapping of peptic digests of labeled and unlabeled enzyme. Observed Y and B series fragments are shown above and below the peptide sequence, respectively. The identification of these peptide fragments, along with the primary sequence of the enzyme, conclusively defines the sequence of the isolated peptide.

with 2-deoxy-2-fluoro- $\alpha$ -L-fucosyl fluoride, the enzyme was diluted into pH 2 buffer. Immediate proteolysis with pepsin was then used to generate peptide fragments of a size that could be analyzed by reverse-phase HPLC/electrospray mass spectrometry. Comparative mapping analysis of peptides from both labeled and native protein digests identified two peptides that eluted at the same retention time (45 min) and had  $m/z$  927.5 and 853, respectively. The  $m/z$  difference of 74.5 between the two corresponds to that expected if the peptide is doubly charged with  $m/z$  853 and carries a label of mass 149 Da, as would be the case with a 2-deoxy-2-fluorofucosyl moiety. The ratio of labeled to unlabeled peptide was estimated to be 3:1. Analysis of all possible peptides by inspection of the enzyme amino acid sequence identified 19 peptides with mass  $1705 \pm 2$  Da. Using the known location of pepsin-specific cleavage sites, only one peptide of this mass,  $^{222}\text{WNDMGWPEKKGEDL}^{235}$ , is expected from pepsin proteolysis.

Information on the sequence of the peptide bearing the label and its site of attachment was obtained in two separate experiments. In the first experiment the peptide of  $m/z$  853 was isolated by reverse phase-HPLC and sequenced by tandem mass spectrometry (Fig. 5). Peaks corresponding to the B ions WN ( $m/z$  301), WND ( $m/z$  416), WNDMG ( $m/z$  604), and WNDMGW ( $m/z$  790) provide sequence information for the N-terminal end of the unlabeled peptide. Peaks arising from Y ions of the unlabeled peptide give information about the C-terminal end; EDL ( $m/z$  376), KEDL ( $m/z$  504), GKEDL ( $m/z$  561), KGKEDL ( $m/z$  689), PEKKGEDL ( $m/z$  915), WPEKKGEDL ( $m/z$  1101), GWPEKKGEDL ( $m/z$  1158), MGWPEKKGEDL ( $m/z$  1289), DMGWPEKKGEDL ( $m/z$  1404), and NDMGWPEKKGEDL ( $m/z$  1518). This information, combined with the mass of the intact peptide and the primary sequence of the enzyme, conclusively confirms the sequence of the labeled peptide to be that predicted. This peptide contains 4 carboxylic acid residues that may bear the 2-fluorofucosyl label. A second MS/MS analysis was performed on the labeled peptide to identify the position of attachment. The fragmentation pattern observed was very similar to that in Fig. 5 but also included a peak at  $m/z$  = 940 (data not shown). This peak at  $m/z$  = 940 corresponds to the peptide fragment  $^{222}\text{WNDMGW}^{227}$  ( $m/z$  791) bearing the fucosyl label (149 Da). The location of the label can

			*
<i>T. maritima</i>	(217)	LPDVLWNDMGWPEK	(230)
<i>X. campestris</i>	(303)	QPDLIYFDWIIAHP	(316)
<i>X. fastidiosa</i>	(299)	APELIYFDWIIAHP	(312)
<i>M. musculus</i>	(209)	KPDLIWSDGWECPC	(222)
<i>C. elegans</i>	(218)	NPEVVWSDGEWDKS	(231)
<i>R. norvegicus</i>	(219)	KPDLIWSDGWECPC	(232)
<i>C. familiaris</i>	(223)	EPDLIWSDGWKCPC	(236)
<i>H. sapiens</i>	(218)	KPDLIWSDGWECPC	(231)

FIG. 6. Alignment of the region containing the proposed catalytic nucleophile aspartate labeled by 2-deoxy-2-fluoro- $\alpha$ -L-fucosyl fluoride in selected glycosyl hydrolases of family 29. The  $\alpha$ -L-fucosidase sequences shown are: *T. maritima* (AAD35394); *Xanthomas campestris* (AAM42160); *X. fastidiosa* (AAF85750); *Mus musculus* (BAB21949); *Rattus norvegicus* (CAA34268); *Canis familiaris* (CAA63362); *Homo sapiens* (AAA52481). The GenBank<sup>TM</sup> accession numbers are in parentheses. The conserved aspartate residue of family 29  $\alpha$ -L-fucosidases is shown in bold.

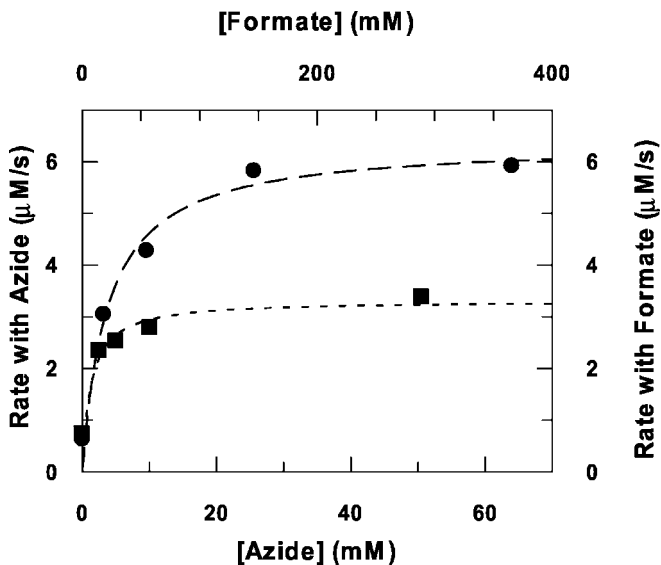


FIG. 7. Chemical rescue of the D224A mutant of the *T. maritima*  $\alpha$ -L-fucosidase. The effect of varying concentrations of azide (●) and formate (■) on the rate of enzyme-catalyzed cleavage is shown. Apparent  $K_m$  values for these anions are  $4 \pm 1$  mM (azide) and  $5 \pm 1$  mM (formate). Reactions were performed at 37 °C, pH 5.0, with pNP-fucoside as substrate.

therefore be deduced because this peptide contains only one carboxylic acid residue, Asp-224, capable of bearing the glycosyl label. Sequence alignments of this peptide with sequences of other glycosidases of family 29 show that this aspartate residue is completely conserved across the family, providing very strong additional evidence for this residue being the catalytic nucleophile (Fig. 6).

**Kinetic Analysis of the D224A Mutant Enzyme**—To demonstrate the importance of this putative catalytic nucleophile residue a site-directed mutant, D224A, was constructed. Production of this mutant as a His<sub>6</sub>-tagged protein proved challenging, but this problem was overcome by expression of the mutant as an MBP fusion protein. To assess the effect of the maltose-binding protein, and thereby allow for a more accurate comparison, the wild-type  $\alpha$ -L-fucosidase was also prepared as an MBP fusion construct and its kinetic parameters with the two substrates determined (Table I). When compared with the His<sub>6</sub>-wild type enzyme, the MBP-wild type fusion protein was found to have a slightly higher  $k_{\text{cat}}$  (~2.6 times greater) against both substrates, with the  $K_m$  remaining similar. This difference in  $k_{\text{cat}}$  is difficult to account for, but does not affect the conclusions of this work. Early studies revealed that the MBP form of

the protein was less stable, thus assays with *p*NP-fucoside were performed at a reduced temperature of 37 °C.

The D224A mutant was tested for catalytic activity using both *p*NP-fucoside and fucosyl fluoride substrates, as shown in Table I. This mutant enzyme showed a greatly reduced activity with *p*NP-fucoside as a substrate compared with wild type enzyme. No saturation was observed at 1 mM substrate concentrations, therefore only a value of  $k_{\text{cat}}/K_m$  could be extracted. Higher substrate concentrations could not be investigated because of the limited solubility of *p*NP-fucoside. The  $10^4$ -fold decrease in  $k_{\text{cat}}/K_m$  observed in this study is substantial, but less than that typically found with nucleophile mutants of  $\beta$ -retaining glycosidases, where a  $10^5$ – $10^8$ -fold decrease is more common (33–35). It is, however, more similar to the  $2.3 \times 10^4$  decrease observed for the nucleophile mutant of another  $\alpha$ -glucosidase: *Bacillus circulans* cyclodextrin glycosyltransferase (36). The residual catalytic activity of this mutant is unlikely to arise from wild type contamination because in that case a dependence on substrate concentration with a similar  $K_m$  to that of the wild type enzyme would be expected. Unfortunately, when this mutant was assayed with fucosyl fluoride, because of the low sensitivity of the fluoride electrode assay and the relatively higher spontaneous hydrolysis rate, no activity of this enzyme above background hydrolysis could be detected using fucosyl fluoride as a substrate ( $<0.01 \text{ s}^{-1}$  at 500  $\mu\text{M}$  fucosyl fluoride). The very low hydrolytic activity observed with this mutant enzyme is entirely consistent with the proposed role of this mutated residue as catalytic nucleophile.

**Chemical Rescue of D224A with *p*NP-fucoside as Substrate**—Further investigation of this mutant was performed to see if catalytic activity could be restored by the addition of exogenous nucleophiles. It is well documented that the mutation to alanine of catalytic residues in glycosidase active sites creates cavities in which small anions can bind (37). In some cases these anions can then restore function to the mutated enzyme by acting in place of the missing residue. In the case of  $\beta$ -retaining glycosidases the return of activity can approach wild type levels (34, 38). The effect on  $\alpha$ -retaining glycosidases on the other hand is often more modest with a 2–10-fold increase being more common (2). Azide and formate were investigated for their ability to restore function to this mutant using *p*NP-fucoside as a substrate. The addition of even a small concentration of azide (0–30 mM) was sufficient to increase the rate of substrate cleavage by up to 10-fold (Fig. 7). The rate increase was seen to be dependent on azide concentration up to 30 mM, whereupon saturation was observed and no further rate increase was observed. Similar data were also obtained with formate, a 5-fold rate increase being observed in this case (Fig. 7). This saturation behavior most likely represents the relatively tight binding of the exogenous anion in the enzyme cavity formed upon removal of the catalytic nucleophile. Azide and formate are observed to bind with similar affinities, with apparent  $K_m$  values of 4 and 5 mM, respectively.

**Conclusion**—By a combination of active site labeling and mutagenesis we have conclusively identified Asp-224 to be the catalytic nucleophile of the *T. maritima*  $\alpha$ -L-fucosidase. This represents the first direct identification of the catalytic nucleophile for any family 29 glycosyl hydrolase. The successful la-

beling of this  $\alpha$ -L-fucosidase with a 2-deoxy-2-fluorosugar, in contrast to the behavior typically seen by this class of inactivator with  $\alpha \rightarrow \alpha$ -D-glycosidases, suggests that there are mechanistic features of this enzyme in common with  $\beta$ -D-glycosidases; a similarity also observed with human  $\alpha$ -L-iduronidase (28).

**Acknowledgment**—Pedro Coutinho is thanked for help with the sequence alignments.

**Note Added in Proof**—During the review process, a paper appeared describing the identification of the equivalent residue as the nucleophile in another family 29  $\alpha$ -L-fucosidase (39).

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