# Probing the Catalytically Essential Residues of the α-L-Fucosidase from the Hyperthermophilic Archaeon *Sulfolobus solfataricus*†

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ABSTRACT: Retaining glycosidases promote the hydrolysis of the substrate by following a double-displacement mechanism involving a covalent intermediate. The catalytic residues are a general acid/base catalyst and the nucleophile. Experimental identification of these residues in a specific glycosidase allows for the assigning of the corresponding residues in all of the other enzymes belonging to the same family. By means of sequence alignment, mutagenesis, and detailed kinetic studies of the α-fucosidase from *Sulfolobus solfataricus* (Ssα-fuc) (family 29), we show here that the residues, invariant in this family, have the function inferred from the analysis of the 3D structure of the enzyme from *Thermotoga maritima* (Tmα-fuc). These include in Ssα-fuc the substrate-binding residues His46 and His123 and the nucleophile of the reaction, previously described. The acid/base catalyst could be assigned less easily. The $k_{cat}$ of the Ssα-fucGlu292Gly mutant, corresponding to the acid/base catalyst of Tmα-fuc, is reduced by 154-fold but could not be chemically rescued. Instead, the Ssα-fucGlu58Gly mutant revealed a 4000-fold reduction of $k_{cat}/K_M$ if compared to the wild-type and showed the rescue of the $k_{cat}$ by sodium azide at wild-type levels. Thus, our data suggest that a catalytic triad, namely, Glu58, Glu292, and Asp242, is involved in catalysis. Glu58 and Glu292 cooperate in the role of acid/base catalyst, while Asp242 is the nucleophile of the reaction. Our data suggest that in glycosidase family 29 α-fucosidases promoting the retaining mechanism with slightly different catalytic machineries coexist.

Glycoside hydrolases are a wide group of enzymes that promote the hydrolysis of the glycosidic bond of the substrate in two different manners: retention and inversion of the anomeric configuration. *Inverting* glycosidases operate with a one step, single-displacement mechanism with the assistance of a general acid and a general base group in the active site. The general acid protonates the glycosidic oxygen to accelerate the reaction, while the base polarizes a water molecule that attacks the anomeric carbon; the resulting product shows an anomeric configuration inverted if compared to the substrate (*1*). Instead, *retaining* enzymes (Scheme 1) follow a two-step mechanism with formation of a covalent glycosyl−enzyme intermediate. The carboxyl group in the active center functions as a general acid/base catalyst, and the carboxylate functions as the nucleophile of the reaction (*2*). In the first step (glycosylation step), the nucleophile attacks the anomeric group of the substrate, while the acid/base catalyst, acting in this step as a general acid, protonates the glycosidic oxygen, thereby assisting the leaving of the aglycon moiety. The concerted action of the two amino acids leads to the formation of a covalent glycosyl−enzyme intermediate. In the second step (deglycosylation step), the glycosyl−enzyme intermediate is cleaved by a water molecule that acts as nucleophile being polarized by the general base catalyst. The product of the reaction retained the anomeric configuration of the substrate.

The identification of key active-site residues in glycosidases is crucial to understand the catalytic mechanism (*1*, *3*), to allow the classification of this class of enzymes (*4*, *5*), and to produce glycoside hydrolases with novel characteristics (*6*). The active-site residues of retaining glycosidases have been identified with a variety of methods including mechanism-based inhibitors, inspection of X-ray crystallographic data, and the combination of amino acid sequence analysis, mutagenesis, and chemical rescue strategies (reviewed in ref *7*). The use of specific inactivators has proven to be of great utility in labeling the catalytic nucleophile (*8*−*11*), whereas a reliable method of labeling the acid/base catalyst is still elusive and successful approaches are less common (*12*, *13*). For these reasons, the acid/base catalyst of several retaining glycosidases was identified through detailed analysis of mutants in which conserved aspartic and glutamic acid residues have been replaced by isosteric and nonionizable amino acids as asparagine, glutamine, alanine, or glycine (*7*).

By following this approach, the catalytic residues of glycoside hydrolase family 29 (GH29)[1] have been recently described. GH29 includes only α-fucosidase enzymes from

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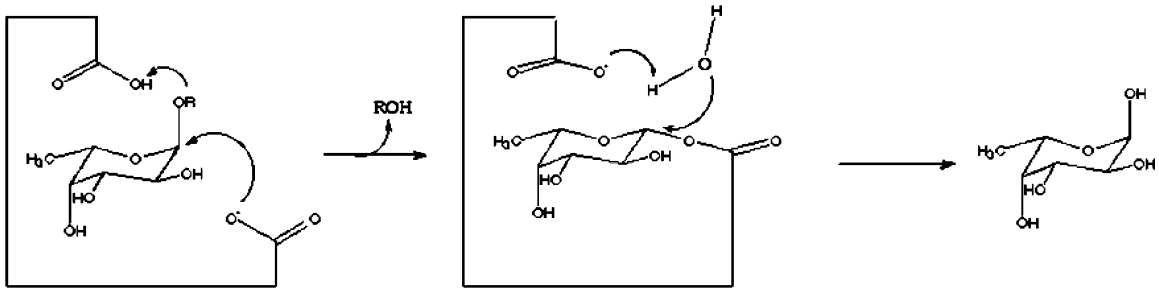
Scheme 1: Reaction Mechanism of a Retaining α-Fucosidase




the three living domains. α-L-Fucosidases (EC 3.2.1.51) are exoglycosidases specific for α-linked L-fucose residues in glycoconjugates. Fucosylated oligosaccharides are involved in a variety of biological processes (*14*, *15*). α-L-Fucosidase activity is diagnostic for several carcinomas (*16*−*18*), and its deficiency causes fucosidosis, a well-known lysosomal disorder (*19*).

The only archaeal member of GH29 is the α-L-fucosidase from the hyperthermophile *Sulfolobus solfataricus* (Ssα-fuc). Ssα-fuc was prepared by site-directed mutagenesis from two open-reading frames (ORFs) separated by a −1 frameshifting cloned from the genome of *S. solfataricus*; a single mutation in the region of overlap between the two ORFs produced a full-length gene that expressed in *Escherichia coli* a fully functional α-L-fucosidase (*20*, *21*). Ssα-fuc follows the retaining reaction mechanism (Scheme 1) (*20*). We identified the nucleophile of the reaction by mutating the invariant Asp242 residue and isolating the product of the reaction obtained by chemical rescue of the activity with the external nucleophile sodium azide (*22*). By following a different approach, but in full agreement with our result, the group of Steve Withers identified the corresponding residue of the α-L-fucosidase from the hyperthermophilic bacterium *Thermotoga maritima* (Tmα-fuc) (*11*). In addition, more recently, the same authors obtained the crystal structure of complexes of Tmα-fuc with α-L-fucose and with a mechanism-based inhibitor. This study allowed the identification of Glu266 as the general acid/base catalyst of the enzyme through the inspection of the 3D structure and the detailed kinetic characterization of active-site mutants (*23*). Remarkably, although Glu266 and Glu66, with the latter being involved in the substrate binding, were both essential for activity, they were not conserved in GH29 and could be identified only through inspection of the 3D structure.

In the present study, we modified by site-directed mutagenesis several residues of Ssα-fuc that, from sequence alignments of GH29 and from the 3D structure of Tmα-fuc, were predicted to be involved in catalysis. We show here that the kinetic characterization of the mutants of the invariant amino acids confirms the role predicted by the Tmα-fuc structure. Instead, intriguingly, the kinetic behavior of the Ssα-fuc mutants of the residues functionally corresponding to Glu66 and Glu266 (acid/base) in Tmα-fuc indicates that in the archaeal enzyme they are both involved in catalysis. This finding supports the hypothesis that in Ssα-fuc the reaction is catalyzed by a catalytic triad rather than the conventional dyad found in the majority of retaining glycosidase. Our data suggest that in GH29 α-fucosidases promoting the retaining mechanism with slightly different catalytic machineries coexist.

## MATERIALS AND METHODS

*Reagents*. All commercially available substrates were purchased from Sigma. The GeneTailor Site-Directed Mutagenesis System was from Invitrogen, and the synthetic oligonucleotides were from Qiagen.

*Sequence Analysis*. GH29 α-fucosidases were downloaded from http://afmb.cnrs-mrs.fr/CAZY/index.html. Redundant sequences may affect the quality of the alignment; thus, in a preliminary screening, we multialigned the sequences of the same organism and we excluded from the subsequent analysis those showing more than 70% identity. The alignment was created with the program Muscle run with its default parameters (http://www.drive5.com/muscle).

*Site-Directed Mutagenesis*. The plasmid pGEX-frameFuc expressing Ssα-fuc (GenBank accession number AY887105) was described previously (*20*). The mutants H46G, E58G, H123G, D124G, D146G, E229G, and E292G were prepared by site-directed mutagenesis from the pGEX-frameFuc plasmid, by following the instructions of the manufacturer. The mutagenic oligonucleotides were the following (mismatches are underlined): H46G, 5′-CTAAATTCGGTATTTTTATCGGTTGGGGAGTATA-3′; E58G, 5′-AGTACCAGCATTTGGTAATGGATGGTACCCTA-3′; H123G, 5′TAGTCCTAGTTGCAGAACATGGCGATGGATTTG-3′; D124G, 5′-GTCCTAGTTGCAGAACATCACGGTGGATTTGCACT-3′; D146G, 5′-CCAAGATTGGACCTAAAAGGGGCATTGTTAGAGA-3′; E229G, 5′-GATTGGTTGCTTAGAATTGTTGGGGCTGTTGAAAA-3′; and E292G, 5′-TATACGTTCTTTATTGTTCCTCTTCCAGCTAAATCC-3′.

In the mutagenic oligonucleotide E292G, a second mismatch was inserted in the codon AGG encoding for Arg293 to disrupt the stretch of C that increases the melting temperature. The resulting change AGG → AGA was a nonsense mutation encoding for the amino acid Arg. The plasmids containing the desired mutation were identified by direct sequencing and completely resequenced.

*Enzymes Preparation and Characterization.* Wild-type and mutant α-fucosidases were expressed and purified as described previously (*20*). The final heating step for all of the mutants was performed at 70 °C. The enzymes resulted in >95% purity by SDS−PAGE. The protein concentration was

[1] Abbreviations: GH29, glycoside hydrolase family 29; Ssα-fuc, α-fucosidase from *S. solfataricus*; Tmα-fuc, α-fucosidase from *Thermotoga maritima*; 4NP-Fuc, 4-nitrophenyl-α-L-fucopyranoside; 4NP-Fuc-Fuc, 4-nitrophenyl-3-*O*-α-L-fucopyranosyl-α-L-fucopyranoside; ORF, open-reading frame; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; TLC, thin-layer chromatography.

determined with the method of Bradford (*24*), by using bovine serum albumin as the standard. The samples stored at 4 °C in 20 mM sodium phosphate buffer at pH 7.0, 150 mM NaCl, and 0.02% sodium azide are stable for several months.

The standard assay of the α-fucosidase activity was performed at 65 °C in 50 mM sodium phosphate buffer at pH 6.3, with the 4-nitrophenyl-α-L-fucopyranoside (4NP-Fuc) substrate at the final concentration of 1 mM as reported previously (*20*). In all of the assays, spontaneous hydrolysis of the substrate was subtracted by using appropriate blank mixtures without the enzyme.

The activity of the E58G mutant rescued by sodium azide and sodium formate was measured by using 0.01−2 M sodium azide or 0.5 M formate, in 50 mM sodium phosphate buffer at pH 6.3 and 1 mM 4NP-Fuc as described previously (*22*). Steady-state kinetic parameters of the wild-type Ssα-fuc were measured as described previously (*20*). Kinetic constants of the mutants were measured at 65 °C by using 4NP-Fuc substrate concentrations ranging from 0.005 to 10 mM, in 0.2 mL of the buffers indicated. The amount of the enzymes used in the assays ranges between 1 and 14 μg. The reaction was started by adding the enzyme, and it was stopped by adding 0.8 mL of 1 M iced sodium carbonate. The optical density of the solution was measured at 420 nm at room temperature. The molar extinction coefficient of 4-nitrophenol, measured at 420 nm, at room temperature and in 1 M sodium carbonate buffer is 17.2 $mM^{-1}$ $cm^{-1}$. At the conditions tested, the reaction was linear in the first 5 min of incubation. All kinetic data were calculated as the average of at least two experiments and were plotted and refined with *GraFit* (*25*). When the enzymes could not be saturated even at the highest concentration of 4NP-Fuc (25 mM), the $k_{cat}/K_M$ values were determined by using the Lineweaver−Burk plot.

Thermal activity and thermal stability measurements of the wild-type and E292G mutant were performed as described previously (*20*). Residual activity of the E58G mutant in thermal activity and thermal stability experiments was measured at the standard conditions with added 150 mM sodium azide.

The pH dependence of the wild-type and mutants was determined by assaying the enzymes at 65 °C, 1 mM 4NP-Fuc, in 50 mM of the following buffer systems: sodium citrate (pH range of 3.0−5.6), sodium phosphate (pH range of 6.0−8.0), and glycine/NaOH (pH range of 8.0−10). The activity was measured after addition of sodium carbonate as described above (*22*).

*Analysis of 2′-Fucosyllactose Hydrolysis by HPAEC.* Wild-type and E58G and E292G mutants were incubated in 50 mM sodium phosphate buffer at pH 6.3, 1 mM 2′-fucosyllactose, at 65 °C, in the absence or presence of 150 mM sodium azide. After incubation, the reactions were stopped in dry ice. Aliquots of the reaction were preparaed for the subsequent analysis by diluting them to 25 μL with water to obtain a final amount of about 1 nmol of sugar and by adding 0.5 nmol of xylose as an internal standard. This step allowed us to load on the column 1.5 nmol of sugars as requested by the manufacturers. The samples were loaded on a CarboPac PA-10 column (Dionex, Sunnyvale, CA) equilibrated with 0.016 M NaOH at pH 11 and equipped with a Spectra Physics AS3000 autosampler. Runs were performed at room temperature at 1 mL/min, and the products were detected with a Dionex PAD II pulsed electrochemical detector, working in the amperometric detection mode and equipped with a gold working electrode and an Ag/AgCl reference electrode. The amount of fucose produced was determined by using a standard curve obtained by injecting samples of fucose at known concentrations. Integration was performed with PC1000 software (Thermo Separation Products).

Table 1: Comparison of Highly Conserved Nucleophilic Residues in the α-Fucosidase Enzymes from *T. maritima* and *S. solfataricus*

| Tmα-fuc residues | Ssα-fuc residues | proposed function in the active site[a] | frequency in GH29 of Ssα-fuc residues[b] (%) |
|---|---|---|---|
| His34 | His46 | 4-hydroxyl | 91 |
| Glu66 | Glu58 | 3-hydroxyl | 40 (3) |
| His128 | His122 | 4-hydroxyl | 93 |
| His129 | His123 | 2-hydroxyl | 88 |
| Asp130 | Asp124 | absent | 89 |
| Asp152 | Asp146 | absent | 95 |
| Glu211 | Glu229 | absent | 91 |
| Asp224 | Asp242 | nucleophile | 96 |
| Glu266 | Glu292 | acid/base | 51 (17) |

[a] Data are from Sulzenbacher and collaborators (*23*); 4-, 3-, and 2-hydroxyl indicate the interaction of the reported amino acid with the corresponding groups in fucose. [b] Frequency was calculated from the alignment reported in Figure 1; for His residues, frequency was calculated on the basis of the number of histidines present in that position of the alignment, while for Asp/Glu residues, frequency takes into account both aspartic and glutamic acids. Data in parentheses indicate the frequency in GH29 of Tmα-fuc residues (see Figure 1).

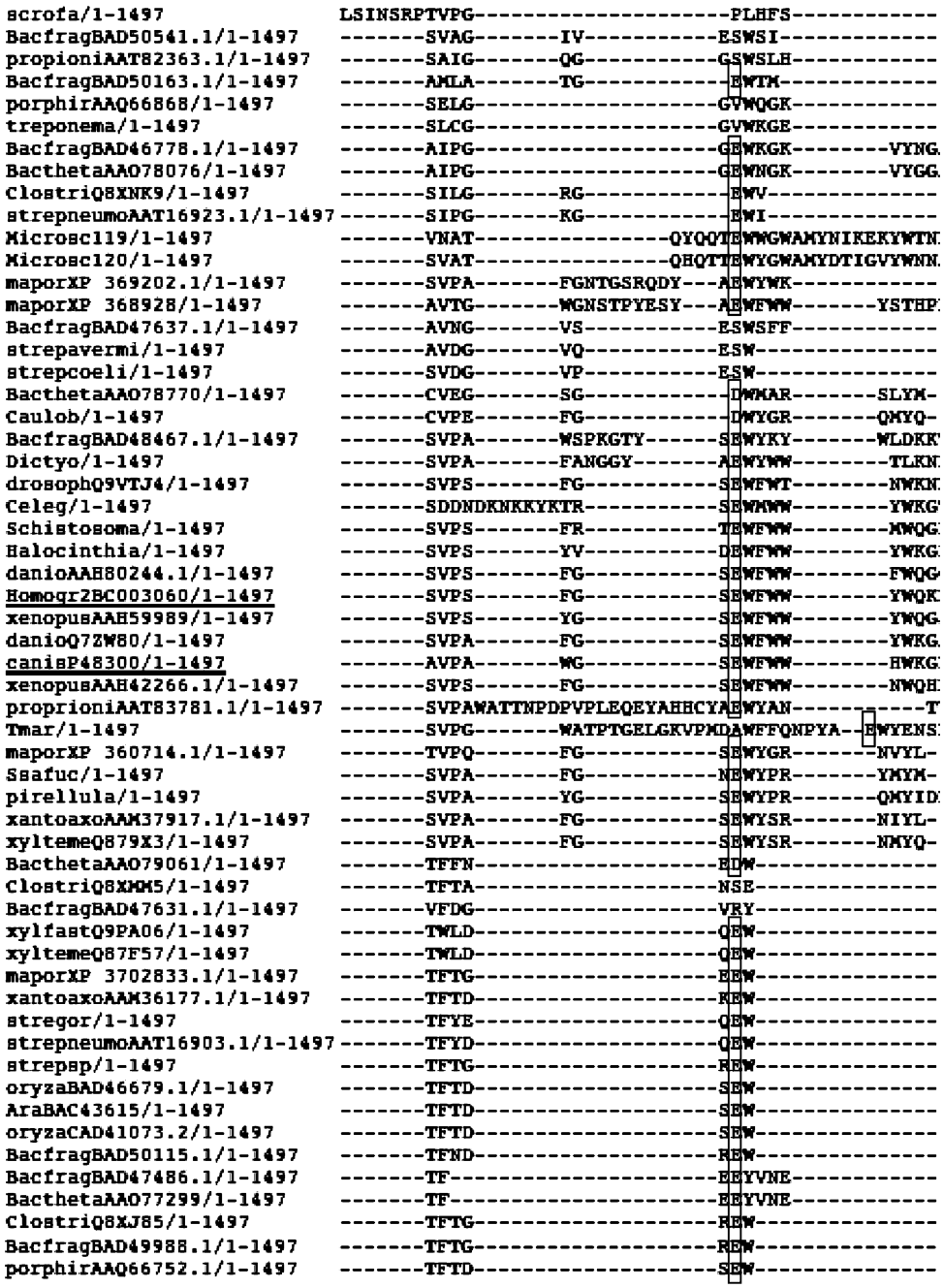
## RESULTS

*Identification of the Active-Site Residues.* We searched the residues putatively involved in the reaction mechanism and substrate binding of Ssα-fuc by analyzing the multialignment of 57 nonredundant GH29 sequences available in the CAZY data bank (http://afmb.cnrs-mrs.fr/CAZY/index.html). From the multialignment, among histidine, aspartic, and glutamic acid residues, we identified seven highly conserved amino acids that may be involved in the reaction: His46, His122, His123, Asp124, Asp146, Glu229, and Asp242 (Ssα-fuc numbering). These residues are mostly invariant in GH29, being present in more than 50 sequences of the 56 analyzed. To find hints on the possible function of these residues, we analyzed the 3D structure of the α-fucosidase from *Thermotoga maritima* (Tmα-fuc) (*23*) (Table 1). His34 and His128, which correspond to His46 and His122 in Ssα-fuc, respectively, stabilize the 4-hydroxyl group of fucose, while His129 (His123 in Ssα-fuc) interacts with the 2-hydroxyl group of the product (Table 1). Because these residues are almost invariant and, presumably, are not involved in catalysis, we decided to analyze His46 and His123 only.

Asp124, Asp146, and Glu229 in Ssα-fuc correspond to Asp130, Asp152, and Glu211 in Tmα-fuc, respectively. These residues are not placed in the active site of the bacterial enzyme; however, because their frequency in GH29 is higher than 95%, they were included in our study. Finally, Asp242, corresponding to Tmα-fuc Asp224, is the nucleophile of the reaction; the role of these residues has been described elsewhere (*11*, *22*).

Interestingly, the residues that play an essential role in catalysis in the α-fucosidase from *T. maritima*, Glu66 and Glu266 (Tmα-fuc numbering) (*23*), fall in regions of the

**A**




alignment scarcely conserved in GH29 (parts A and B of Figure 1), precluding an unequivocal identification of the corresponding residues in *S. solfataricus* α-fucosidase. Thus, we used the program Muscle, which is specifically suitable for sequences with <30% identities (*26*), to perform a pairwise alignment of Ssα-fuc and Tmα-fuc (Figure 1C). The inspection of the alignment revealed that in Ssα-fuc Glu58 and Glu292 corresponded to Tmα-fuc Glu66 and Glu266, respectively. We included these residues in our analysis.

To study the role played in catalysis by the identified residues of Ssα-fuc, we mutated them in the nonnucleophilic amino acid glycine. The resulting mutants were H46G, E58G, H123G, D124G, D146G, E229G, and E292G; they were expressed and purified as previously reported for the wild-type Ssα-fuc by using an efficient purification step by affinity chromatography on gluthatione-sepharose (*22*). To get rid of the possible source of contamination with the wild-type enzyme, a question that plagues any detailed analyses of enzyme mutants with expected low activity (*27*), we used new samples of the gluthatione-sepharose matrix for each mutant purified.

Preliminary enzymatic assays revealed that the E229G mutant was not affected in its thermophilicity and enzymatic activity. This amino acid is highly conserved in GH29 (Table 1), and in Tmα-fuc, it corresponds to Glu211 that is located in the α-helix A3, distant from the active center (*23*). These issues indicate that Glu229 is not involved in catalysis; therefore, the mutant E229G was not further characterized.

In Table 2, we report the kinetic constants of the wild-type and mutant α-fucosidases at 65 °C in 50 mM sodium phosphate buffer at pH 6.3 on the 4-nitrophenyl-α-L-fucopyranoside substrate (4NP-Fuc). The mutants D124G and D146G show small differences in their catalytic activity and substrate specificity, resulting in a 3.7- and 1.5-fold reduction of their catalytic efficiency, respectively, if com-

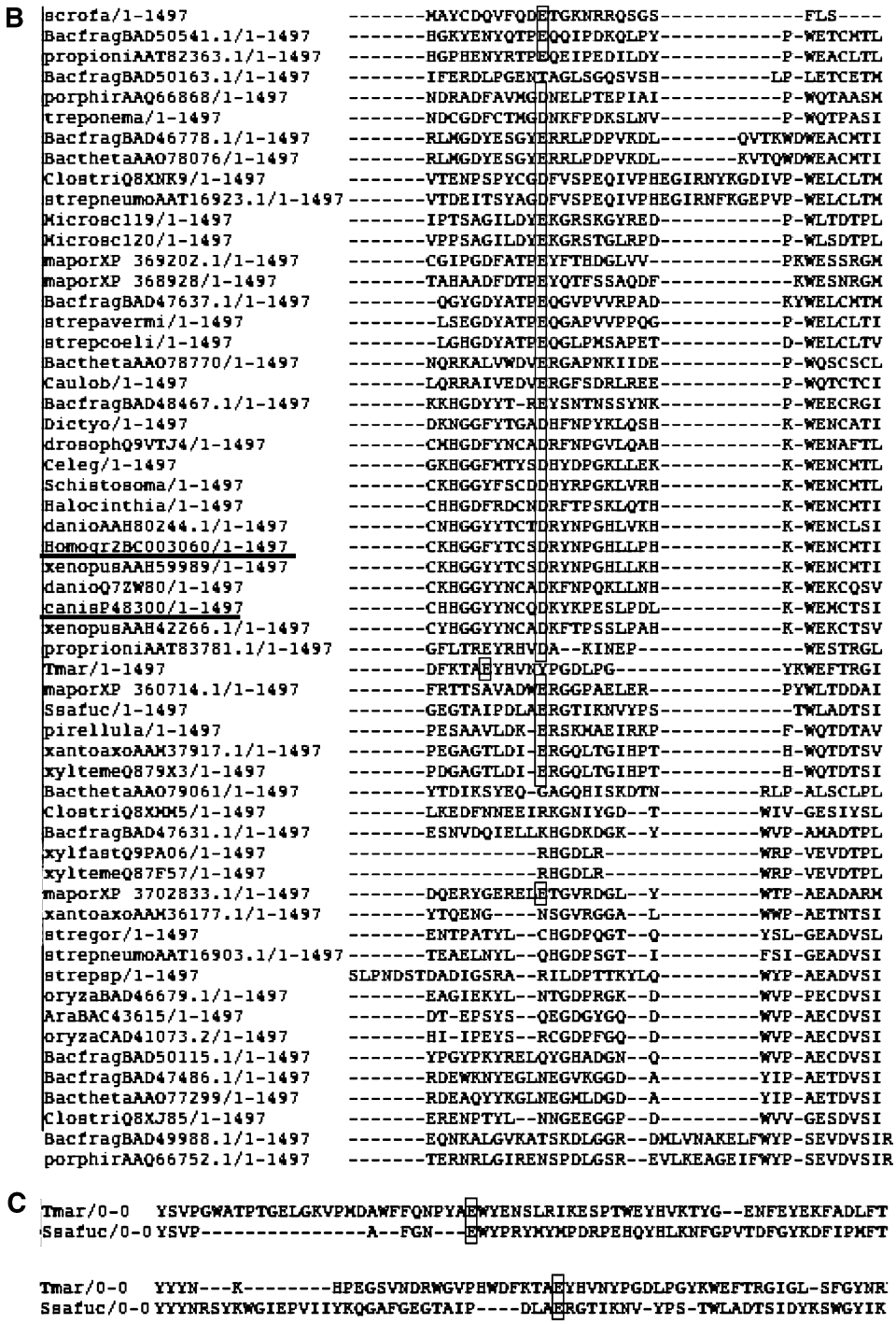



FIGURE 1: Alignment of nonredundant GH29 amino acid sequences. The Asp/Glu residues corresponding to Glu58 (A) and Glu292 (B) are boxed. As a comparison, Tmα-fuc Glu66 and Glu266 are also boxed in A and B, respectively. The α-fucosidases from human and dog are underlined. The pairwise alignment of Tmα-fuc and Ssα-fuc sequences is reported in C. The two sequences showed an overhall 29% identity. The matching residues Glu66/Glu58 and Glu266/Glu292 (Tmα-fuc/Ssα-fuc numbering) are boxed and shown in the upper and lower part of C, respectively. AraBAC43615, *Arabidopsis thaliana*; oryzaCAD41073.2 and oryzaBAD46679.1, *Oryza sativa*; stregor, *Streptococcus gordonii V288*; strepneumoAAT16903.1 and strepneumoAAT16923.1, *Streptococcus pneumoniae*; ClostriQ8XJ85, ClostriQ8XNK9, and ClostriQ8XMM5, *Clostridium perfrigens 13*; BacfragBAD49988.1, BacfragBAD47486.1, BacfragBAD50115.1, BacfragBAD48467.1, BacfragBAD47637.1, BacfragBAD50541.1, BacfragBAD46778.1, BacfragBAD47633101, and BacfragBAD50163.1, *Bacteroides fragilis YCH46*; porphirAAQ66752.1 and porphirAAQ66868, *Porphiromonas gingivalis W83*; BacthetaAA077229, BacthetaAA078770, BacthetaAA079061, and BacthetaAA078076, *Bacteroides thetaiotaomicron VPI-5482*; xantoaxoAAM36177.1 and xantoaxoAAM37917.1, *Xanthomonas axonopodis pv. citri str. 306*; maporXP_3702833.1, moporXP_369202.1, maporXP_368928, and maporXP_360714.1, *Magnaporthe grisea 70−15*; xylfastQ9PA06, *Xylella fastidiosa 9a5c*; xyltemeQ87F57 and xyltemeQ879X3, *Xylella fastidiosa temecula1*; sptrepsp, *Streptomyces* sp. 142; pirellula, *Pirellula* sp. 1; Ssαfuc, *Sulfolobus solfataricus P2* (*20*); Caulob, *Caulobacter crescentus CB15*; Microsc119 and Microsc120, *Microscilla* sp. *PRE1*; Tmar, *Thermotoga maritima*; propioniAAT83781.1 and propioniAAT82363.1, *Propionibacterium acnes KPA171202*; canisP48300, *Canis familiaris*; xenopusAAH42266.1 and xenopusAAH59989, *Xenopus laevis*; danioAAH80244.1 and danioAAH80244.1, *Danio rerio*; Homogr2BBC003060, *Homo sapiens*; halocinthia, *Halocynthia roretzi*; drosophQ9VTJ4, *Drosophila melanogaster*; schistosoma, *Schistosoma japonicum*; celeg, *Caernorhabditis elegans*; dictyo, *Dictyostelium discoideum*; strepavermi, *Streptomyces avermitilis MA-4680*; strepcoeli, *Streptomyces coelicolor A3(2)*; treponema, *Treponema denticola ATCC 35405*; scrofa, *Sus scrofa*.

Table 2: Steady-State Kinetic Constants of Wild-Type and Mutants α-Fucosidases[a]

| | $k_{cat}$ ($s^{-1}$) | $K_M$ (mM) | $k_{cat}/K_M$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|
| wild-type | 287 ± 11 | 0.028 ± 0.004 | 10 250 |
| D124G | 240 ± 7 | 0.088 ± 0.012 | 2740 |
| D146G | 224 ± 5 | 0.033 ± 0.003 | 6791 |
| H46G | 419 ± 99 | 17.0 ± 5.7 | 25 |
| H123G[b] | ND[c] | ND | 5.96 |
| E58G[b] | ND | ND | 2.63 |
| E292G | 1.86 ± 0.09 | 0.056 ± 0.013 | 33 |

[a] Assays were performed at 65 °C in 50 mM sodium phosphate buffer at pH 6.3 on 4NP-Fuc. [b] No saturation observed on up to 25 mM 4NP-Fuc; the specificity constants were calculated from the Lineweaver−Burk plots (see the Materials and Methods). [c] Not determined.

pared to that of the wild-type. Moreover, the specific activity of these mutants increased with temperature as observed for the wild-type, with an optimal temperature higher than 80 °C (not shown). These data confirm the inspection higher than Tmα-fuc 3D structure, indicating that the aspartic acids 124 and 146 are not directly involved in catalysis. Therefore, the mutants were not characterized further.

Instead, the affinity for the substrate of H46G and H123G was remarkably different from that of the wild-type: the mutation of His46 produced a 607-fold increase of the $K_M$, while no saturation was observed with the mutant H123G at the highest concentrations of 4NP-Fuc used. Also the mutation of the residues Glu58 and Glu292 affected catalysis severely (Table 2); again, no saturation could be observed with the former residue, while E292G showed unchanged affinity for 4NP-Fuc but a 154-fold reduction in the turnover number. The data reported in Table 2 clearly indicate that residues His46, His123, Glu58, and Glu292 are involved in catalysis or in the substrate binding; hence, we characterized the mutants in great detail.

*Characterization of H46G and H123G Mutants.* The effect on the $K_M$ of the mutation of His46 and His123 indicates that these residues could be involved in substrate recognition and binding. This is confirmed by the kinetic constants of H46G and H123G mutants in 50 mM sodium acetate buffer at pH 5.0 that are compared to those obtained at pH 6.3 in Table 3. No significant differences were found with H123G that, still, at pH 5.0, could not be saturated. Instead, the affinity for the substrate of the H46G mutant increased slightly, and the catalytic efficiency was 4.5-fold higher than that calculated at pH 6.3. This result contrasts with that of the wild-type whose $k_{cat}/K_M$ at pH 5.0 is reduced by 6.3-fold as a result of the almost 10-fold increase in $K_M$.

The analysis of the pH dependence of the H46G and H123G mutants confirmed the reduced specific activity of the enzymes at all of the pH values tested in the range of 3.5−10; however, the dependence from the pH and the pH optimum of both mutants were identical to those of the wild-type (not shown). This observation and the lack of chemical rescue of the activity of the two mutants in the presence of up to 2 M sodium azide (not shown) further confirm that His46 and His123 are not involved in catalysis, thereby indicating that they are involved in substrate recognition.

*Characterization of E58G and E292G Mutants.* The analysis of the 3D structure of the Tmα-fuc and the kinetic characterization of the mutants clearly indicate that, in this enzyme, Glu66 and Glu266 (Tmα-fuc numbering) are involved in substrate binding and in the acid/base catalysis, respectively (*23*). As described in Figure 1C, the corresponding residues in Ssα-fuc are Glu58 and Glu292 and their mutation produced a dramatic effect on the catalytic constants (Table 2), suggesting that these residues may be involved in catalysis. However, intriguingly, the lack of saturation observed for the mutant E58G reflects the result observed for the mutation of Glu266, the acid/base catalyst of Tmα-fuc (*23*). Instead, the 154-fold reduction in the $k_{cat}$ of the E292G mutant, whose $K_M$ is unaltered and similar to that of the wild-type, suggests its involvement in the acid/base catalysis (Table 2). However, the kinetic analysis of these did not allow us to unequivocally determine whether Glu58 or Glu292 is the acid/base catalyst.

A diagnostic tool for the identification of the acid/base catalyst is the comparison of the pH dependence of the wild-type and mutants (*7*); therefore, we analyzed the effect of pH on the reaction catalyzed by E58G and E292G mutants (Figure 2A). The pH behavior of wild-type Ssα-fuc below pH 3.0 could not be examined because of instability of the enzyme in this pH range; however, the basic limb of the curve is peculiar and did not allow the calculation of the p$K_a$ (Figure 2A). The specific activity of the E292G mutant is very low at all of the pH values tested (Figure 2A); however, when the data are reported as a percent of the maximal activity versus pH, this mutant revealed a behavior similar to that of the wild-type (Figure 2B). In contrast, the E58G mutant, although showing higher residual activity at pH 4−6 (Figure 2A), follows a bell-shaped curve strikingly different from that of the wild-type with a maximum at pH 4.6 (Figure 2B). The shape of the curve allows the calculation of the ionization at the basic limb corresponding to a p$K_a$ of 5.3.

The most important diagnostic tool for the identification of the acid/base catalyst in retaining glycosidases is the chemical rescue of the enzymatic activity of the mutants (*7*, *28*−*33*). Therefore, we analyzed the ability of sodium azide and sodium formate to restore the activity of E58G and E292G on the 4NP-Fuc substrate. The specific activity of the mutant E58G at 65 °C in 50 mM sodium phosphate buffer at pH 6.3 increased by 71- and 80-fold in the presence of 2 M sodium azide and 0.5 M sodium formate, respectively, on 1 mM 4NP-Fuc, while the activation was of 52-fold in 1 M NaCl on 6 mM substrate. The maximal activity of the E58G mutant on 1 mM 4NP-Fuc is observed at 150 mM sodium azide, and the enzyme shows a Michaelis−Menten-like dependence of the reaction rate on the concentration of azide with an apparent $K_M$ of 28 ± 8 mM (Figure 2C). Similarly, the maximal rate in the presence of sodium formate was observed at 500 mM; hence, in all of the subsequent characterizations, we used a concentration of azide and formate ions of 150 and 500 mM, respectively.

In striking contrast, the external nucleophiles did not produce any effect on the specific activity of the E292G mutant (not shown); these results contrast with those obtained with the E266A mutant of Tmα-fuc, whose activity was rescued by externally added ions (*23*). As a control reaction, the wild-type Ssα-fuc is activated by 2 M sodium azide only by 1.6-fold. Our results indicate that in Ssα-fuc the chemical rescue is effective only when the Glu58 side chain is removed by mutation, suggesting that this residue acts as the acid/base catalyst.

Table 3: Steady-State Kinetic Constants of Wild-Type and H46G and H123G Mutants at Different pHs

| | pH 5.0[a] | | | pH 6.3[b] | | |
|---|---|---|---|---|---|---|
| | $k_{cat}$ ($s^{-1}$) | $K_M$ (mM) | $k_{cat}/K_M$ ($s^{-1}$ $mM^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_M$ (mM) | $k_{cat}/K_M$ ($s^{-1}$ $mM^{-1}$) |
| wild-type | 430 ± 49 | 0.26 ± 0.09 | 1,624 | 287 ± 11 | 0.028 ± 0.004 | 10,250 |
| H46G | 495 ± 27 | 4.37 ± 0.71 | 113 | 419 ± 99 | 17.0 ± 5.7 | 25 |
| H123G[c] | ND[d] | ND | 34 | ND | ND | 5.96 |

[a] Assays were performed at 65 °C in 50 mM sodium acetate buffer at pH 5.0 on 4NP-Fuc. [b] Assays were performed at 65 °C in 50 mM sodium phosphate buffer at pH 6.3 on 4NP-Fuc. [c] No saturation observed with up to 25 mM 4NP-Fuc; the specificity constants were calculated from the Lineweaver−Burk plots (see the Materials and Methods). [d] Not determined.

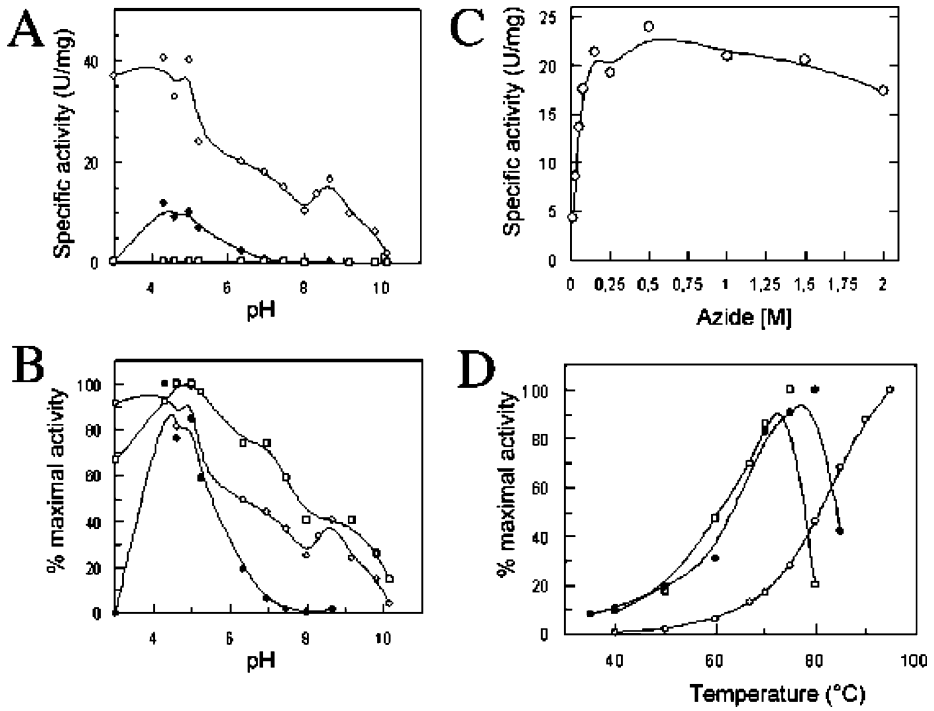



FIGURE 2: Enzymatic characterization of wild-type, E58G, and E292G α-fucosidases. Dependence on pH is reported as specific activity (A) or percent of the maximal activity (B) on 4NP-Fuc at 65 °C as reported in the Materials and Methods. The wild-type is reported as ○, and E58G and E292G are reported as ● and □, respectively. The data are the average of at least three experiments, with the standard deviation being about 5%. (C) Dependence of the activity of E58G on sodium azide. (D) Thermal activity of wild-type, E58G, and E292G α-fucosidases. The data are the average of at least three experiments, with the standard deviation being about 5%.

To test if the reduced activity of the E292G mutant was due to destabilization, we analyzed the effect of temperature on the stability and activity of the two mutants. The E58G and E292G mutants show reduced thermal activity if compared to the wild-type, showing an optimal temperature of 75 °C followed by a drop of activity at higher temperatures (Figure 2D). These results presumably reflect the lower stability of the mutants; in particular, E58G is fully active for 10 min at 65 °C, while it shows half-lives of 6.2 and 1.3 min at 75 and 80 °C, respectively. Instead, E292G has half-lives of 7 h, 49 min, and 5.6 min at 65, 75, and 85 °C, respectively. As a comparison, the wild-type Ssα-fuc is fully active for 2 h at 65 °C and maintains 60% residual activity after 2 h at 80 °C (*20*). Nevertheless, the two mutants were stable enough at 65 °C, the temperature at which we performed the kinetic characterization described above. This allowed us to exclude that the reduced activity of the E292G mutant was the effect of destabilization.

The chemical rescue of mutants in the acid/base catalyst of retaining glycosidases determines the production of glycosyl-azide products showing the same anomeric configuration of the substrate (*7*). The analysis of the products of the reaction of the E58G mutant in the presence of 300 mM sodium azide and 11 mM 4NP-Fuc in 50 mM sodium phosphate buffer at pH 7.0 and 65 °C did not reveal by TLC analysis any product corresponding to fucosyl-azide (not shown). However, interestingly, the mutant catalyzed the synthesis of 4-nitrophenyl-3-*O*-α-L-fucopyranosyl-α-L-fucopyranoside (4NP-Fuc-Fuc) with a yield of 15%,[2] indicating that it followed a retaining mechanism.

To test how external nucleophiles affect catalysis, we measured the kinetic constants of the wild-type and mutant Ssα-fuc enzymes in different buffer systems in the pH range of 4.6−6.3 (Table 4). The activity of the wild-type was not increased by formate and acetate (not shown), while the $k_{cat}$ at pH 5.0 in sodium citrate buffer was slightly higher (1.5-fold) than that in sodium phosphate at pH 6.3. As observed with the azide, the kinetic constants of the E292G mutant remained unchanged in acetate, phosphate (Table 4), and citrate (not shown). Instead, remarkably, the activity of the E58G mutant is rescued in the presence of sodium citrate, acetate, and formate buffers at pH 4.6 (Table 4). In particular,

[2] The product was purified and identified by comparison with the NMR spectra of the product obtained by the transfucosylation reaction of the wild-type Ssα-fuc described previously (*20*).

Table 4: Steady-State Kinetic Constants of Wild-Type and E58G and E292G Mutants in Different Buffers

| | $k_{cat}$ ($s^{-1}$) | $K_M$ (mM) | $k_{cat}/K_M$ ($s^{-1}$ $mM^{-1}$) | reaction conditions[a] | pH |
|---|---|---|---|---|---|
| wild-type | 287 ± 11 | 0.028 ± 0.004 | 10 250 | sodium phosphate | 6.3 |
| | 430 ± 49 | 0.26 ± 0.09 | 1624 | sodium citrate | 5.0 |
| E58G | ND[b] | ND | 2.63 | sodium phosphate | 6.3 |
| | 143 ± 8 | 1.6 ± 0.3 | 89 | sodium citrate | 4.6 |
| | 586 ± 43 | 0.62 ± 0.18 | 950 | sodium acetate | 4.6 |
| | 846 ± 46 | 1.07 ± 0.18 | 790 | sodium formate | 4.6 |
| | 679 ± 32 | 2.96 ± 0.43 | 229 | sodium phosphate + $NaN_3$[c] | 6.3 |
| E292G | 1.86 ± 0.09 | 0.056 ± 0.013 | 33 | sodium phosphate | 6.3 |
| | 1.86 ± 0.17 | 0.087 ± 0.024 | 21.3 | sodium acetate | 4.6 |

[a] Assays were performed at 65 °C in the buffers reported in 50 mM concentrations on 4NP-Fuc. [b] Not determined; no saturation was observed with up to 25 mM 4NP-Fuc. The specificity constant was calculated from the Lineweaver−Burk plots (see the Materials and Methods). [c] Sodium azide was used at the concentration of 150 mM.

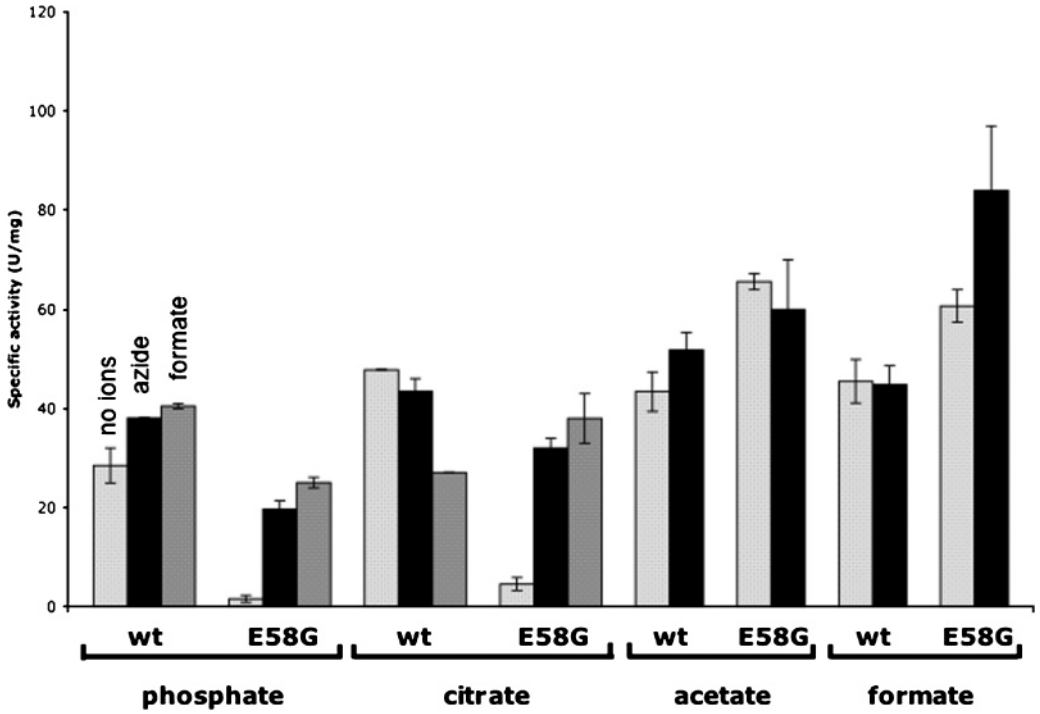



FIGURE 3: Chemical rescue experiments. The activity of the enzymes was measured in 50 mM sodium phosphate at pH 6.3, 50 mM sodium citrate at pH 4.6, 50 mM sodium acetate at pH 4.6, and 50 mM sodium formate at pH 4.6 on 4NP-Fuc at 65 °C (light gray bars). The specific activity obtained when 0.5 M sodium formate or 150 mM sodium azide were added to the reaction mixture is indicated in dark gray and black bars, respectively.

in the presence of these buffers, we observed the substrate saturation of the mutant and we could calculate a $K_M$ value. More interestingly, the $k_{cat}$ increases enormously in the order citrate < acetate < formate, resulting in the $k_{cat}$ increasing up to 3-fold higher than that of the wild-type. A similar effect is observed with externally added 150 mM sodium azide in sodium phosphate buffer at pH 6.3 (Table 4): again, E58G was saturated by 4NP-Fuc, and the rate constant observed was 2.4-fold higher than that of the wild-type.

Noticeably, the chemical rescue of E58G occurs at pH 4.6 in different buffers and at pH 6.3 in the presence of azide; therefore, the reactivation observed is not solely a pH effect, but rather, it depends on the nature of the ions used. These results, which mirror the observations reported previously with other acid/base mutants of retaining glycosidases (*7*), further support the hypothesis that in Ssα-fuc Glu58 is the acid/base catalyst.

To investigate the mechanism of the activating external nucleophiles, we tested if they have a combined effect on the activity of the mutants. To this aim, we assayed wild-type and mutant enzymes in different buffers in the presence of 150 mM sodium azide or 0.5 M sodium formate (Figure 3). Interestingly, the mutant E58G was activated by both nucleophiles in the presence of 50 mM sodium phosphate buffer at pH 6.3 and 50 mM sodium citrate buffer at pH 4.6; instead, in 50 mM sodium acetate at pH 4.6 and 50 mM sodium formate at pH 4.6, sodium azide had a limited effect (Figure 3). Remarkably, however, in acetate and formate, the specific activity of E58G was higher than that of the wild-type enzyme, whose activity did not change significantly at all of the conditions used. In striking contrast, the specific activity of the E292G mutant was unaltered (about 0.25 ± 0.02 unit/mg) at all of the conditions tested (not shown).

These data suggest that azide, formate, and acetate ions activate E58G on 4NP-Fuc by occupying the cavity created by the mutation and working as nucleophiles. Instead, the ion citrate, being bigger, may have limited access because of space and/or charge hindrance in the active site, thereby showing limited activation of the mutant (see the Discussion and Table 4).

To shed light on the catalytic step modified by the external ions, we analyzed the specific activity of wild-type and mutants on 2′-fucoyllactose. This compound is a substrate of the wild-type Ssα-fuc, showing a group (lactose) with worse leaving ability if compared to 4-nitrophenol of 4NP-Fuc. Hence, the limiting step of the hydrolysis of 2′-fucosyllactose is the glycosylation of the enzyme.

The mutant E58G was completely inactive on this substrate; i.e., the amount of fucose detected by HPAEC analysis after incubation at 65 °C in 50 mM sodium phosphate buffer at pH 6.3 did not vary in the experimental conditions and was approximately the same as in the control without the enzyme. Instead, the mutant E292G showed very low but detectable specific activity (0.37 unit/mg) when assayed at the same conditions, suggesting that other catalytic groups still present in E292G can promote the glycosylation step of the reaction. Remarkably, the activity of the E58G mutant on 2′-fucosyllactose could be chemically rescued with added 150 mM sodium azide; at these conditions, the mutant has a specific activity of 2.8 units/mg, a value similar to that obtained with the wild-type assayed at optimal conditions (3.3 units/mg). In contrast, the specific activity of the E292G was not rescued by azide, resulting in 0.44 unit/mg.

## DISCUSSION

All known genes encoding for α-L-fucosidases belong to family 29 of the glycoside hydrolase classification. Recently, a large amount of data became available on enzymes from this family; in particular, by studying the α-fucosidase from the archaeon *S. solfataricus*, we reported for the first time that GH29 followed a retaining reaction mechanism and we unequivocally assigned to Asp242 the nucleophile of the reaction (*20*, *22*). These data were confirmed by Tarling and collaborators who identified the nucleophile of the α-fucosidase from the bacterium *T. maritima* (*11*). More recently, the determination of the 3D structure of Tmα-fuc and the characterization of active-site mutants allowed the assignment to Glu266 (Tmα-fuc numbering) the role of the acid/base catalyst (*23*).

Once the nucleophile and general acid/base catalyst have been unequivocally identified in a particular family, the glycoside hydrolase classification allows us to easily pick the corresponding residues in any enzyme belonging to that particular family. In fact, because catalytic residues are generally essential, they are invariant in the family and can be identified by amino acid sequence alignments. This method is extremely consistent and explains the utility of the carbohydrate active enzyme classification (http://afmb-.cnrs-mrs.fr/CAZY/index.html) that is widely used.

By following this line of approach, we decided to analyze the residues involved in the catalytic activity and substrate binding of Ssα-fuc. As expected, the residue acting as the nucleophile of the reaction in GH29, Asp224 and Asp242 in Tmα-fuc and Ssα-fuc, respectively, is almost invariant (frequency of 98% in Table 1). Similarly, we have shown here, by kinetic characterization of the corresponding mutants, that other residues that are highly conserved in GH29, namely, His46, His122, and His123 in Ssα-fuc, are involved in substrate binding as predicted by the crystal structure of Tmα-fuc complexed to α-L-fucose (*23*). Thus, the reduced affinity for the substrate of Ssα-fucH46G and Ssα-fucH123G mutants (Table 2) and the absence of reactivation by external ions clearly support the hypothesis that these residues interact with the hydroxyl groups bound to C4 and C2 of the substrate.

In contrast, the acid/base catalyst of Tmα-fuc, Glu266, and another residue, Glu66, which is also located in the active site of the enzyme, are conserved to a lesser extent in GH29. In particular, the frequencies of Glu66 anf Glu266 in this family are 3 and 14%, respectively (Table 1). However, a pairwise alignment of Ssα-fuc and Tmα-fuc showed that these residues corresponded to Ssα-fuc Glu58 and Glu292 (Figure 1C).

To determine whether Glu58 or Glu292 was the acid/base catalyst, we followed the classical approach in which the glutamic acids are replaced by glycine and the obtained mutants are kinetically characterized.

The first diagnostic tool to test if the general acid/base has been removed by mutation is the analysis of the steady-state kinetic constants; in fact, it has been pointed out that, if the acid/base of a retaining glycosidase is removed, this affects both steps of the reaction, but the effect depends on the substrate used (*7*). In the case of poor substrates, showing groups with worse leaving ability (aglycons with $pK_a > 8$), the first step of the reaction (glycosylation) requires the participation of the general acid/base catalyst. Instead, for good substrates (aglycons with $pK_a < 8$), the glycosylation step needs less assistance and the second step of the reaction (deglycosylation) is dependent on the assistance of the acid/base catalyst that functions as a general base at this stage (Scheme 1) (*28*, *30*, *34*). In our study, we used 4NP-Fuc as the substrate; the $pK_a$ of the leaving group 4-nitrophenol (7.18) suggests that the limiting step in the hydrolysis is at the borderline between glycosylation and deglycosylation. Hence, without the analysis of the activity of the enzyme on substrates with different leaving groups, it is not possible to establish in advance which is the limiting step of the reaction of Ssα-fuc with the 4NP-Fuc substrate.

The kinetic characterization of the Ssα-fucE58G and Ssα-fucE292G mutants gave unexpected results; in fact, the lack of saturation on the 4NP-Fuc substrate of Ssα-fucE58G mirrors that of the acid/base mutant in *T. maritima* enzyme (Tmα-fucE266A) (*23*). Instead, Ssα-fucE292G showed the classical behavior of a mutated acid/base catalyst with a 150-fold reduction of $k_{cat}$ and a $K_M$ almost unchanged (Table 2). In fact, the leaving ability of 4NP-Fuc could be good enough to complete the glycosylation step; therefore, when the acid/base catalyst is removed, the $k_{cat}$ value, assessing the deglycosylation step, is significantly reduced. The reduction in the catalytic rate ($k_{cat}$) results in accumulation of the fucosyl−enzyme intermediate and consequently determines a decrease of the $K_M$ value (*7*). Presumably, the $K_M$ did not decrease in Ssα-fucE292G because the $K_M$ of the wild-type is already very low. It is worth noting that the removal of the residue acting as the acid/base catalyst in Tmα-fuc produced a mutant that could not be saturated with 4NP-Fuc (*23*). This behavior has never been reported in retaining glycosidases and may suggest that the catalytic machinery of α-L-fucosidase may differ from those known so far.

A second diagnostic tool for the assessment of the acid/base catalyst is the pH dependence. Typically, glycosidases have a bell-shaped pH dependence, and when the acid/base is removed, the basic limb is severely affected (*7*). Wild-

type Ssα-fuc has a peculiar pH dependence showing a reproducible increase of activity at pH 8.6 (Figure 2A). This behavior suggests that more than two ionizable groups are involved in catalysis (*35*). Instead, for Tmα-fuc, the pH dependence of the wild-type and the Glu266Ala mutant has never been described.

The low specific activity at different pH values of Ssα-fucE292G suggests that Glu292 could be the acid/base catalyst, as observed in other retaining glycosidases (*28*, *29*, *33*, *34*). However, the pH profile resulted in a large change in the case of the Ssα-fucE58G mutant, producing a typical bell-shaped curve with a pH optimum at 4.6 sharper than that of the wild-type (3.0−5.0) (Figure 2B). These data suggest that the removal of Glu58 unmasked the ionization of a group responsible for the basic limb (p$K_a$ = 5.3) and possibly increased the p$K_a$ of the nucleophile of the reaction mainly determining the acidic limb. Hence, these experiments did not allow the assignment of the acid/base catalyst.

The most definite tool to determine the acid/base catalyst is the chemical rescue of the activity of the mutants by nucleophilic anions. Replacing the acid/base catalyst with the small nonionizable glycine residue generally reduces dramatically the activity of the mutant. In addition, the presence of the glycine results in sufficient space in the active site so that a small nucleophile can be accommodated in this cavity after the formation of the glycosyl−enzyme intermediate. In these cases, rate enhancement and the isolation of a glycosyl-azide product with the same anomeric configuration of the substrate are expected (*7*). Activity rate enhancements by means of external nucleophiles resulted in several cases, including Tmα-fuc, the most effective method to unequivocally identify the acid/base catalyst (*13*, *23*, *27*−*36*). Remarkably, Ssα-fucE58G was activated by more than 70-fold in the presence of sodium azide, formate, acetate, and chloride (Table 4 and Figure 3). In particular, it is worth noting that in the presence of sodium acetate and sodium formate the $k_{cat}$ was even higher than that of the wild-type (Table 4). In striking contrast, the activity of Ssα-fucE292G could not be rescued by any of the nucleophiles used; this result makes it very unlikely that Glu292 is the acid/base catalyst of Ssα-fuc, and our data would assign this role to Glu58.

Unfortunately, from the reaction of Ssα-fucE58G, we could not isolate the α-fucosyl-azide product that is expected after the reactivation of an α-fucosidase depleted of the acid/base catalyst. Nevertheless, the detection of the 4NP-Fuc-Fuc product clearly indicates that the mutant followed a retaining reaction mechanism, confirming that the chemical rescue of the Ssα-fucE58G mutant did not perturb the structure of the active site. The same methodology applied to the mutant in the nucleophile of the reaction. Ssα-fucD242G produced only trace amounts of the expected β-fucosyl-azide (*22*); thus, presumably, in the case of Ssα-fucE58G, the product could have been lost during the purification. Alternatively, azide may act not as a nucleophile but as a base, polarizing water, or a substrate molecule working as acceptors. Similar results were obtained with other glycosidases mutated in the acid/base: the mutant of the human α-amylase did not produce the expected product (*27*), and the mutant of a β-glucosidase from *Flavobacterium meningosepticum* formed β-glucosyl-azide and glucose with a 1:4 ratio (*34*). In Tmα-fuc, the analysis of the products of the chemical rescue of the Glu266Ala mutant was not described.

We found further evidence supporting the hypothesis that Glu58 is the acid/base catalyst by assaying Ssα-fucE58G in different buffer systems in the presence of sodium azide (Figure 3). The nucleophile anion activated the mutant only in the presence of larger ions (phosphate and citrate), while this effect was much reduced in acetate and formate in which Ssα-fucE58G is already activated. Presumably, in phosphate and citrate buffers, azide has full access to the small cavity created by the mutation in the active site. Instead, acetate and formate, occupying this space, could activate the reaction and preclude the access to azide.

We obtained unexpected results by analyzing the effect of the azide on the Ssα-fucE58G mutant assayed on 2′-fucosyllactose. For this substrate, the glycosylation is certainly the limiting step because the lactose group has worse leaving ability and requires the assistance of the acid catalyst. Quite unexpectedly, added azide rescued the activity of Ssα-fucE58G at wild-type levels, while Ssα-fucE292G was not reactivated by the external ion displaying basal activity on this substrate. The reactivation of the Ssα-fucE58G mutant would suggest that azide acted as an acid catalyst, assisting the departure of the leaving group in the glycosylation step of the reaction. How this can happen cannot be easily explained, considering that at the pH of the reaction (6.3) sodium azide (p$K_a$ = 4.72) is almost completely ionized. However, Viladot and collaborators suggested that azide might also intervene at the glycosylation step as an enzyme-bound $HN_3$ activating the acid/base mutant of a 1,3−1,4-β-glucanase from *Bacillus licheniformis* (*36*). Alternatively, azide may induce a conformational change in Ssα-fucE58G, recruiting another residue, which can now serve as an acid catalyst.

The low activity of the Ssα-fucE292G mutant on 2′-fucosyllactose further indicates that Glu292 is not the acid/base catalyst, showing that this mutant possesses enough catalytic machinery to promote the glycosylation step.

The sum of our data suggests that in Ssα-fuc a catalytic triad, namely, Glu58, Glu292, and Asp242, is involved in catalysis. This is not unusual in glycoside hydrolases. In the GH11 xylanase from *Bacillus circulans* and in the GH39 β-xylosidase from *Thermoanaerobacterium saccharolyticum*, this case has been studied in great detail, showing that two residues cooperate in catalysis by "reverse protonation" (*13*, *37*). Instead, in a cyclodextrin glycosyl transferase from *B. circulans*, a xylanase from *Aspergillus niger*, and in α-amylases from pig, human, and *A. niger*, a second carboxylic group, adjacent to the acid/base catalyst, modulates the p$K_a$ of the latter, thereby affecting the pH optimum of the enzymes (*38*−*42*).

Intriguingly, the behavior of the catalytic residues of Ssα-fuc is different from that of Tmα-fuc for which the 3D structure is already available (*23*). Nevertheless, considering that among the amino acid sequences of GH29 the predicted acid/base residues are not invariant, it would not be surprising that the enzymes show structural differences in the active site explaining the different catalytic machineries. In particular, Tmα-fuc shows insertions flanking the residues Glu66 and Glu266 (Figure 1C) corresponding to disordered

loops in the 3D structure that may be absent in Ssα-fuc.[3] Thus, to understand the reaction mechanisms of Ssα-fuc at the molecular level requires the analysis at high resolution of 3D-structure complexes of the enzyme with bound ligands.

The presence of glycosidase families with different catalytic residues is not uncommon: acidic xylanases of GH11 perform catalysis with a triad exhibiting a "reverse protonation" mechanism, while alkaline enzymes have a conventional catalytic dyad (*37*). Our evidence suggests that also in GH29 slightly different catalytic machineries coexist. Interestingly, human and canine α-L-fucosidases, whose deficiency causes fucosidosis, show glutamic and aspartic acid residues exactly in the same positions of Ssα-fuc Glu58 and Glu292, respectively (parts A and B of Figure 1). Therefore, the archaeal enzyme could be a useful model system to study the catalytic machinery of the enzymes from mammals.

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## REFERENCES

1. McCarter, J. D., and Withers, S. G. (1994) Mechanisms of enzymatic glycoside hydrolysis, *Curr. Opin. Struct. Biol. 4*, 885−8892.
2. Koshland, D. E. (1953) Stereochemistry and the mechanism of enzymatic reactions, *Biol. Rev. Camb. Philos. Soc. 28*, 416−436.
3. Zechel, D. L., and Withers, S. G. (2000) Glycosidase mechanisms: Anatomy of a finely tuned catalyst, *Acc. Chem. Res. 33*, 11−18.
4. Henrissat, B., and Bairoch, A. (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities, *Biochem. J. 293*, 781−788.
5. Henrissat, B., and Davies, G. (1997) Structural and sequence-based classification of glycoside hydrolases, *Curr. Opin. Struct. Biol. 7*, 637−644.
6. Perugino, G., Trincone, A., Rossi, M., and Moracci, M. (2004) Oligosaccharide synthesis by glycosynthases, *Trends Biotechnol. 1*, 31−37.
7. Ly, H. D., and Withers, S. G. (1999) Mutagenesis of glycosidases. *Annu. Rev. Biochem. 68*, 487−522.
8. McCarter, J. D., and Withers, S. G. (1996) Unequivocal identification of Asp-214 as the catalytic nucleophile of *Saccharomyces cerevisiae* α-glucosidase using 5-fluoro glycosyl fluorides. *J. Biol. Chem. 271*, 6889−6894.
9. Vocadlo, D. J., Mayer, C., He, S., and Withers, S. G. (2000) Mechanism of action and identification of Asp242 as the catalytic nucleophile of *Vibrio furnisii N*-acetyl-β-D-glucosaminidase using 2-acetamido-2-deoxy-5-fluoro-α-L-idopyranosyl fluoride, *Biochemistry 1*, 117−126.
10. Vocadlo, D. J., Davies, G. J., Laine, R., and Withers, S. G. (2001) Catalysis by hen egg-white lysozyme proceeds via a covalent intermediate, *Nature 412*, 835−838.
11. Tarling, C. A., He, S., Sulzenbacher, G., Bignon, C., Bourne, Y., Henrissat, B., and Withers, S. G. (2003) Identification of the catalytic nucleophile of the family 29 α-L-fucosidase from *Thermotoga maritima* through trapping of a covalent glycosyl−enzyme intermediate and mutagenesis, *J. Biol. Chem. 278*, 47394−47399.
12. Tull, D., Burgoyne, D. L., Chow, D. T., Withers, S. G., and Aebersold, R. (1996) A mass spectrometry-based approach for probing enzyme active sites: Identification of Glu 127 in *Cellulomonas fimi* exoglycanase as the residue modified by *N*-bromoacetyl cellobiosylamine, *Anal. Biochem. 234*, 119−125.
13. Vocadlo, D. J., Wicki, J., Rupitz, K., and Withers, S. G. (2002) A case for reverse protonation: Identification of Glu160 as an acid/base catalyst in Thermoanaerobacterium saccharolyticum β-xylosidase and detailed kinetic analysis of a site-directed mutant, *Biochemistry 41*, 9736−9746.
14. Vanhooren, P. T., and Vandamme, E. J. (1999) L-Fucose: Occurrence, physiological role, chemical, enzymatic, and microbial synthesis, *J. Chem. Technol. Biotechnol. 74*, 479−497.
15. Staudacher, E., Altmann, F., Wilson, I. B., and Marz, L. (1999) Fucose in *N*-glycans: From plant to man, *Biochim. Biophys. Acta 1473*, 216−236.
16. Abdel-Aleem, H., Ahmed, A., Sabra, A. M., Zakhari, M., Soliman, M., and Hamed, H. (1996) Serum α-L-fucosidase enzyme activity in ovarian and other female genital tract tumors, *Int. J. Gynaecol. Obstet. 55*, 273−279.
17. Ishizuka, H., Nakayama, T., Matsuoka, S., Gotoh, I., Ogawa, M., Suzuki, K., Tanaka, N., Tsubaki, K., Ohkubo, H., Arakawa, Y., and Okano T. (1999) Prediction of the development of hepatocellular-carcinoma in patients with liver cirrhosis by the serial determinations of serum α-L-fucosidase activity, *Intern. Med. 38*, 927−931.
18. Fernandez-Rodriguez, J., Ayude, D., de la Cadena, M. P., Martinez-Zorzano V. S., de Carlos, A., Caride-Castro, A., de Castro, G., and Rodriguez-Berrocal, F. J. (2000) α-L-Fucosidase enzyme in the prediction of colorectal cancer patients at high risk of tumor recurrence, *Cancer Detect. Prev. 24*, 143−149.
19. Michalski, J. C., and Klein, A. (1999) Glycoprotein lysosomal storage disorders: α- and β-Mannosidosis, fucosidosis, and α-*N*-acetylgalactosaminidase deficiency, *Biochim. Biophys. Acta 1455*, 69−84.
20. Cobucci-Ponzano, B., Trincone, A., Giordano, A, Rossi, M., and Moracci, M. (2003) Identification of an archaeal α-L-fucosidase encoded by an interrupted gene. Production of a functional enzyme by mutations mimicking programmed −1 frameshifting, *J. Biol. Chem. 278*, 14622−14631.
21. Rosano, C., Zuccotti, S., Cobucci-Ponzano, B., Mazzone, M., Rossi, M., Moracci, M., Petoukhov, M. V., Svergun, D. I., and Bolognesi, M. (2004) Structural characterization of the nonameric assembly of an Archaeal α-L-fucosidase by synchrotron small-angle X-ray scattering, *Biochem. Biophys. Res. Commun*, *320*, 176−182.
22. Cobucci-Ponzano, B., Trincone, A., Giordano, A., Rossi, M., and Moracci, M. (2003) Identification of the catalytic nucleophile of the family 29 α-L-fucosidase from *Sulfolobus solfataricus* via chemical rescue of an inactive mutant, *Biochemistry 42*, 9525−9531.
23. Sulzenbacher, G., Bignon, C., Nishimura, T., Tarling, C. A., Withers, S. G., Henrissat, B., and Bourne, Y. (2004) Crystal structure of *Thermotoga maritima* α-L-fucosidase. Insights into the catalytic mechanism and the molecular basis for fucosidosis, *J. Biol. Chem. 279*, 13119−13128.
24. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248−254.
25. Leatherbarrow, R. J. (1992) GraFit version 3.0, Erithacus Software Ltd., Staines, U.K.
26. Edgar, R. G. (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput, *Nucleic Acids Res. 32*, 1792−1797.
27. Rydberg, E. H., Li, C., Maurus, R., Overall, C. M., Brayer, G. D., and Withers, S. G. (2002) Mechanistic analyses of catalysis in human pancreatic α-amylase: Detailed kinetic and structural studies of mutants of three conserved carboxylic acids, *Biochemistry 41*, 4492−4502.
28. MacLeod, A. M., Tull, D., Rupitz, K., Warren, R. A., and Withers, S. G. (1996) Mechanistic consequences of mutation of active site carboxylates in a retaining β-1,4-glycanase from *Cellulomonas fimi*, *Biochemistry 35*, 13165−13172.
29. Bravman, T., Belakhov, V., Solomon, D., Shoham, G., Henrissat, B., Baasov, T., and Shoham, Y. (2003) Identification of the catalytic residues in family 52 glycoside hydrolase, a β-xylosidase from *Geobacillus stearothermophilus* T-6, *J. Biol. Chem. 278*, 26742−26749.

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30. Vallmitjana, M., Ferrer-Navarro, M., Planell, R., Abel, M., Ausin, C., Querol, E., Planas, A., and Perez-Pons, J. A. (2001) Mechanism of the family 1 $\beta$-glucosidase from *Streptomyces* sp: Catalytic residues and kinetic studies, *Biochemistry 40*, 5975−5982.
31. Shallom, D., Belakhov, V., Solomon, D., Gilead-Gropper, S., Baasov, T., Shoham, G., and Shoham, Y. (2002) The identification of the acid−base catalyst of α-arabinofuranosidase from *Geobacillus stearothermophilus* T-6, a family 51 glycoside hydrolase, *FEBS Lett. 514*, 163−167.
32. Zechel, D. L., Reid, S. P., Stoll, D., Nashiru, O., Warren, R. A., and Withers, S. G. (2003) Mechanism, mutagenesis, and chemical rescue of a $\beta$-mannosidase from *Cellulomonas fimi*, *Biochemistry 42*, 7195−7204.
33. Paal, K., Ito, M., and Withers, S. G. (2004) *Paenibacillus* sp. TS12 glucosylceramidase: Kinetic studies of a novel sub-family of family 3 glycosidases and identification of the catalytic residues, *Biochem. J. 378*, 141−149.
34. Li, Y. K., Chir, J., Tanaka, S., and Chen, F. Y. (2002) Identification of the general acid/base catalyst of a family 3 $\beta$-glucosidase from *Flavobacterium meningosepticum*, *Biochemistry 41*, 2751−2759.
35. Debeche, T., Bliard, C., Debeire, P., and O'Donohue, M. J. (2002) Probing the catalytically essential residues of the α-L-arabinofuranosidase from *Thermobacillus xylanilyticus*, *Protein Eng. 1*, 21−28.
36. Viladot, J. L., de Ramon, E., Durany, O., and Planas, A. (1998) Probing the mechanism of Bacillus 1,3-1,4-$\beta$-D-glucan 4-glucanohydrolases by chemical rescue of inactive mutants at catalytically essential residues, *Biochemistry 37*, 11332−11342.
37. Joshi, M. D., Hedberg, A., and McIntosh, L. P. (1997) Complete measurement of the p$K_a$ values of the carboxyl and imidazole groups in *Bacillus circulans* xylanase, *Protein Sci. 12*, 2667−2670.
38. Strokopytov, B., Penninga, D., Rozeboom, H. J., Kalk, K. H., Dijkhuizen, L., and Dijkstra, B. W. (1995) X-ray structure of cyclodextrin glycosyltransferase complexed with acarbose. Implications for the catalytic mechanism of glycosidases, *Biochemistry 34*, 2234−2240.
39. Krengel, U., and Dijkstra, B. W. (1996) Three-dimensional structure of Endo-1,4-$\beta$-xylanase I from *Aspergillus niger*: Molecular basis for its low pH optimum, *J. Mol. Biol. 263*, 70−78.
40. Qian, M., Haser, R., Buisson, G., Duee, E., and Payan, F. (1994) The active center of a mammalian α-amylase. Structure of the complex of a pancreatic α-amylase with a carbohydrate inhibitor refined to 2.2 Å resolution, *Biochemistry 33*, 6284−6294.
41. Brayer, G. D., Luo, Y., and Withers, S. G. (1995) The structure of human pancreatic α-amylase at 1.8 Å resolution and comparisons with related enzymes, *Protein Sci. 9*, 1730−1742.
42. Boel, E., Brady, L., Brzozowski, A. M., Derewenda, Z., Dodson, G. G., Jensen, V. J., Petersen, S. B., Swift, H., Thim, L., and Woldike, H. F. (1990) Calcium binding in α-amylases: An X-ray diffraction study at 2.1 Å resolution of two enzymes from *Aspergillus*, *Biochemistry 29*, 6244−6249.

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