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Identification of the Catalytic Nucleophile of the Family 29 α-L-Fucosidase from *Sulfolobus solfataricus* via Chemical Rescue of an Inactive Mutant†

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Received June 16, 2003; Revised Manuscript Received July 7, 2003

ABSTRACT: We have recently reported that a functional α-L-fucosidase could be expressed by a single insertional mutation in the region of overlap between the ORFs SSO11867 and SSO3060 of the hyperthermophilic Archaeon *Sulfolobus solfataricus* [Cobucci-Ponzano et al. *J. Biol. Chem.* (2003) *278*, 14622−14631]. This enzyme, belonging to glycoside hydrolase family 29 (GH29), showed micromolar specificity for *p*-nitrophenyl-α-L-fucoside (pNp−Fuc) and promoted transfucosylation reactions by following a reaction mechanism in which the products *retained* the anomeric configuration of the substrate. The active site residues in GH29 enzymes are still unknown. We describe here the identification of the catalytic nucleophile of the reaction in the α-L-fucosidase from *S. solfataricus* by reactivation with sodium azide of the mutant Asp242Gly that shows a 10^3-fold activity reduction on pNp−Fuc. The detailed stereochemical analysis of the fucosyl-azide produced by the mutant reactivated on pNp−Fuc revealed its inverted (beta-fucosyl azide) configuration compared with the substrate. This allows for the first time the unambiguous assignment of Asp242, and its homologous residues, as the nucleophilic catalytic residues of GH29 α-L-fucosidases. This is the first time that this approach is used for α-L-glycosidases, widening the applicability of this method.

α-L-Fucosidases (3.2.1.51) are exoglycosidases capable of cleaving α-linked L-fucose residues from glycoconjugates involved in a variety of biological processes (*1*). In particular, the determination of α-fucosidase activity can be used to predict the development of several carcinomas (*2*−*4*), whereas the deficiency in this enzyme causes fucosidosis, a well-known lysosomal storage disorder (*5*).

We have recently reported that the first α-L-fucosidase from an Archaeon, the hyperthermophile *Sulfolobus solfataricus* strain P2, could be expressed in *Escherichia coli* from the ORFs SSO11867 and SSO3060 (*6*). These ORFs encode polypeptides homologous to the N- and the C-terminal fragments, respectively, of α-fucosidase enzymes of family 29 (GH29)[1] (*7*). The two ORFs are separated by a −1 frameshift and this leads to a truncated product. However, by inserting a single base by site-directed mutagenesis in the region of overlap between SSO11867 and SSO3060, we obtained a full-length thermophilic and thermostable α-L-fucosidase. This enzyme, named Ssα-fuc, showed a k_{cat}/K_M of 10 250 s^{-1} mM^{-1} on pNp−Fuc and promoted transfucosylation reactions by following a *retaining* reaction mechanism (*6*). Remarkably, the mutation was designed on the basis of the programmed −1 frameshifting mechanism (*8*); therefore, the functionality of the full-length enzyme gave support to the hypothesis that translational *recoding* events, known so far only in Eukarya and Bacteria (*9*), could be used to regulate gene expression in Archaea.

Our data unequivocally demonstrate that GH29 enzymes follow a *retaining* reaction mechanism. Retaining enzymes typically have two catalytic carboxylic acids in their active site and utilize a double displacement mechanism (Scheme 1) in which a β-L-fucosyl intermediate is formed and hydrolyzed. Both steps proceed via transition states with substantial oxocarbenium ion character (*10*). One carboxylic

† This work was supported by Agenzia Spaziale Italiana project: "Extremophilic Archaea as model systems to study origin and evolution of early organisms: molecular mechanisms of adaptation to extreme physical-chemical conditions" contract no. I/R/365/02 and by MIUR project "Folding di proteine: l'altra metà del codice genetico".

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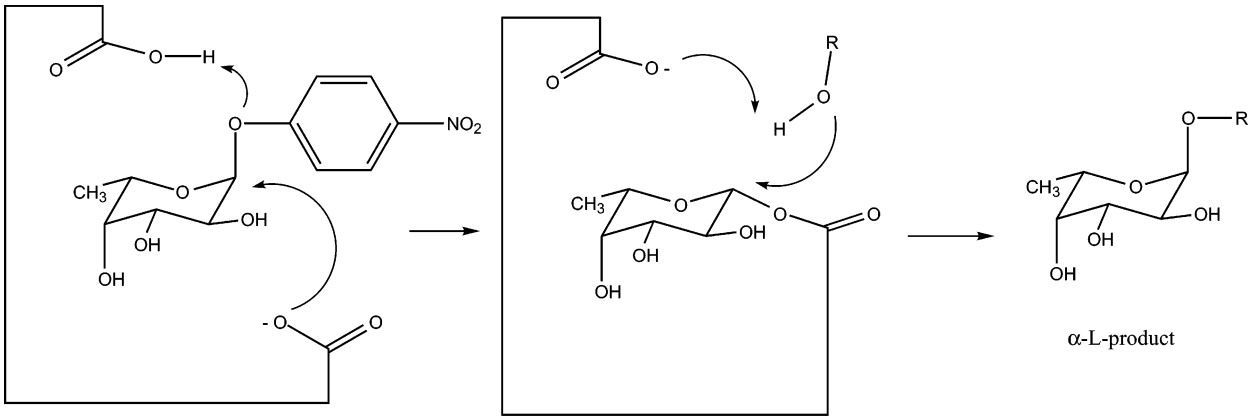
[1] Abbreviations: GH29, glycoside hydrolase family 29; Ssα-fuc, α-fucosidase from *S. solfataricus*; pNp−Fuc, *para*-nitrophenyl-α-L-fucopyranoside; ORF, open reading frame; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; COSY, correlation spectroscopy; FT-IR, Fourier transform infrared spectroscopy; GST, glutathione *S*-transferase; Rf, ratio factor.

10.1021/bi035036t CCC: $25.00
Published on Web 07/25/2003

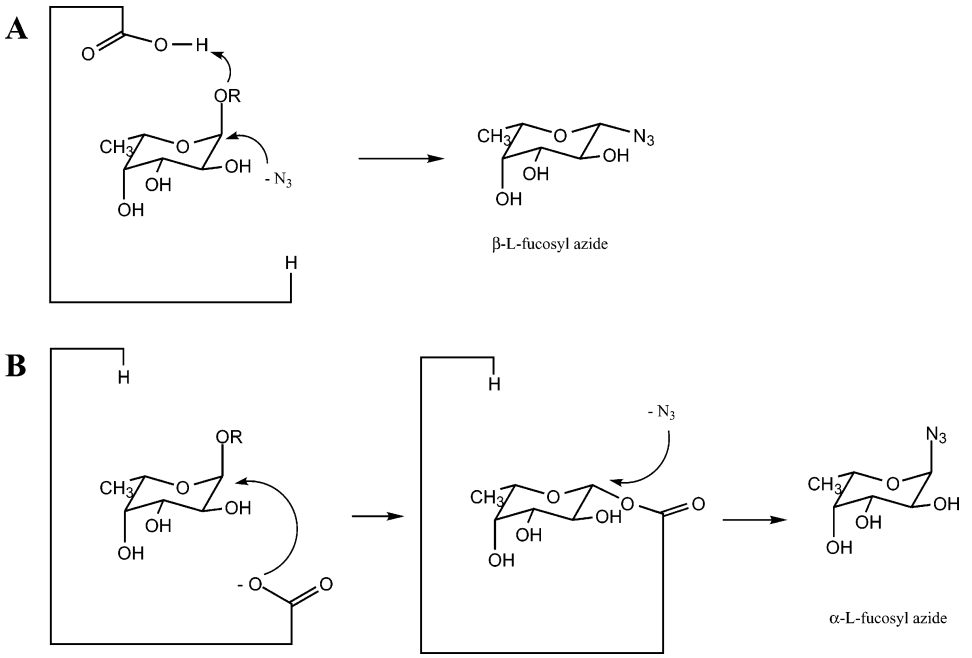
Scheme 1



R = H ⟶ hydrolysis

R = Acceptor ⟶ transfucosylation

Scheme 2



acid in the active site acts as the catalytic nucleophile, leading to the formation of the covalent intermediate, while the other plays the role of the general acid catalyst in the first step and the general base catalyst in the second step of the reaction.

The active site residues in GH29 enzymes are still unknown. For the identification of the catalytic nucleophile in retaining α-D and *β*-D-glycosidases, fluorosugars have proved to be useful mechanism-based inhibitors (*11*), by trapping the glycosyl−enzyme intermediate. This approach involving 5-fluoroglycosides has been employed previously to identify the catalytic nucleophiles of α-D-glucosidases in GH13 and GH31 (*12*, *13*), α-D-galactosidases in GH27 (*14*), and α-D-mannosidases in GH38 (*15*, *16*). Interestingly, a 2-deoxy-2-fluoro sugar has been successfully used recently for the identification of the catalytic nucleophile of a α-L-iduronidase (*17*). An alternative approach often exploited for retaining glycoside hydrolases consists of the mutation of aspartic/glutamic acid residues identified by sequence analysis and conserved in the family of interest. Mutations of the catalytic residues with non-nucleophilic amino acids lead to the strong reduction or even abolition of the enzymatic activity (*18*). However, these mutants can be reactivated in the presence of external nucleophiles as sodium azide. The isolation of glycosyl-azide products with an anomeric configuration opposite to that of the substrate allows the identification of the catalytic nucleophile of the reaction (Scheme 2A) (*18*). By contrast, reactivated mutants in the acid/base catalyst produce glycosyl-azide with the same anomeric configuration of the substrate (Scheme 2B). It is worth noting that this approach has been exploited so far only for *β*-D-retaining glycosidases, as recently reported for GH51 α-L-arabinofuranosidases/*β*-D-xylosidases (*19*) and GH52 *β*-D-xylosidases (*20*), whereas it has never been used for α-(D/L)-retaining glycosidases.

This report describes the identification of the catalytic nucleophile of a known archaeal GH29 α-L-fucosidase by reactivation with sodium azide of a strongly inactivated mutant and by analysis of the anomeric configuration of the fucosyl-azide isolated from the reaction mixture. This is the first time that this approach is used for α-L-glycosidases, showing that it can be of general applicability for retaining enzymes.

MATERIALS AND METHODS

Reagents. All commercially available substrates were purchased from Sigma. The GeneTailor site-directed mutagenesis system was from Invitrogen, and the synthetic oligonucleotide was from Genenco (Italy).

Site-Directed Mutagenesis. The plasmid pGEX-frameFuc expressing Ssα-fuc was described previously (*6*). The mutant Asp242Gly was prepared by site-directed mutagenesis from the pGEX-frameFuc plasmid, by following the instructions of the manufacturer. The mutagenic oligonucleotide carrying the mutation GAC → GGC was the following (mismatch is underlined):

D242G: 5′-GGCCATGGCTAGTCTATTTCGGCTGGTG-GATTGC −3′

The plasmid containing the desired mutation was identified by direct sequencing and completely resequenced.

Enzyme Preparation and Characterization. Wild type and mutant α-fucosidase were expressed and purified as described previously (*6*). The enzymes resulted >95% pure by SDS−PAGE. Protein concentration was determined with the method of Bradford (*21*), by using bovine serum albumin as the standard. The samples stored at 4 °C in sodium phosphate buffer 20 mM pH 7.0, NaCl 150 mM, 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 6.5, with pNp−Fuc substrate at the final concentration of 1 mM as reported previously (*6*). In all the characterization, spontaneous hydrolysis of the substrate was subtracted by using appropriate blank mixtures without enzyme. One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of substrate in 1 min at the conditions described.

The activity of the Asp242Gly mutant at standard conditions was measured by using up to 20 μg of enzyme. The activity of the Asp242Gly mutant rescued by azide was measured in 0.2 mL of 50 mM sodium phosphate buffer, pH 6.5, sodium azide at the concentrations indicated, 2 mM pNp−Fuc, and mutant enzyme (5 μg). The reaction was started by adding the enzyme, and, after 5 min of incubation at 65 °C, it was stopped by adding 0.8 mL of iced sodium carbonate 1 M. The optical density of the solution was measured at 420 nm at room temperature. The molar extinction coefficient of *para*-nitrophenol, measured at 420 nm, at room temperature, and in 1 M sodium carbonate buffer is 17.2 mM^{-1} cm^{-1}. The activity of the Asp242Gly mutant in the presence of sodium formate/formic acid buffer, at the concentrations indicated, and in the pH range 4.0−6.0, was measured in 0.2 mL final volume, at 65 °C, in 2 mM pNpFuc by using Asp242Gly enzyme up to 20 μg. The activity was measured after addition of sodium carbonate as described above for sodium azide. At the conditions tested, the reaction was linear in the first 5 min of incubation. Steady-state kinetic parameters of the wild-type Ssα-fuc were measured as described previously (*6*). Kinetic constants of the Asp242Gly mutant were measured by following the same method described above and by using pNp−Fuc substrate concentrations ranging from 0.05 to 4 mM and from 0.05 to 6 mM for sodium azide and sodium formate, respectively. All kinetic data were calculated as the average of at least two experiments and were plotted and refined with the program GraFit (*22*).

Thermal activity of the wild type was performed as described previously (*6*). Thermal activity of Asp242Gly mutant in the presence of 2 M sodium azide was analyzed by assaying the enzyme (6 μg) as described above on pNp−Fuc substrate concentrations of 2 and 3 mM in the temperature ranges of 40−65 °C and 70−90 °C, respectively.

Isolation of β-L-fucosyl azide from enzymatic reaction mixture of the mutant in 2 M sodium azide was performed by reverse-phase column chromatography and preparative TLC ($CHCl_3$/MeOH/H_2O, 65:25:4 by vol). Acetylation of the eluted material from silica gel was conducted overnight at room temperature in pyridine/Ac_2O. NMR studies (Bruker instrument, 400 MHz, ^1H, COSY and ^1H-^{13}C correlation) of the acetylated product were conducted by dissolving it in $CDCl_3$ using this solvent signal as internal reference standard. ^1H (^{13}C) NMR signal assignments: 4.58 H1 (J = 8.6 Hz) (89.7 C1); 5.14 H2 (68.19 C2); 5.03 H3 (70.99 C3); 5.27 H4 (69.80 C4); 3.90 H5 (71.48 C5); 1.25 H6 (15.88 C6). FT-IR spectrum of the acetylated product shows a diagnostic (N_3) peak at 2119 cm^{-1}.

RESULTS AND DISCUSSION

The enzyme Ssα-fuc expressed in *E. coli* by a single insertional mutation, as recently described (*6*), will be considered the wild type in this paper. To identify the residues involved in catalysis in Ssα-fuc, which are unknown in GH29, we multialigned its amino acid sequence with other non-redundant ORFs from GH29 (Figure 1). Because of the essential role played in catalysis by the nucleophile of the reaction, this residue is generally completely conserved in glycoside hydrolases of the same family. The multialignment produced only four highly conserved aspartic/glutamic acid residues, corresponding in Ssα-fuc to Asp124, Asp146, Glu229, and Asp242. However, in GH29, both Asp and Glu residues are present in the position corresponding to Asp124 and Glu229 of Ssα-fuc (Figure 1). Instead, only Asp146 and Asp242 are invariant aspartic acid residues in all members of the family, strongly suggesting their catalytic role. In fact, in all the glycoside hydrolase families but one (GH90), in which the active site residues were experimentally determined, the catalytic nucleophile/base and the proton donor correspond to invariant Asp or Glu residues (http://afmb.cnrs-mrs.fr/CAZY/index.html).

The residue Asp242 was changed into glycine by site-directed mutagenesis to completely remove the side chain of the aspartic acid. The Asp242Gly mutant is expressed fused to the glutathione *S*-transferase (GST) enzyme as reported previously for the wild-type Ssα-fuc (*6*), and we could purify the enzyme by a single purification step by affinity chromatography. As reported for the wild-type enzyme, the mutant was separated from GST by thrombin cleavage onto the column and was eluted >95% pure.

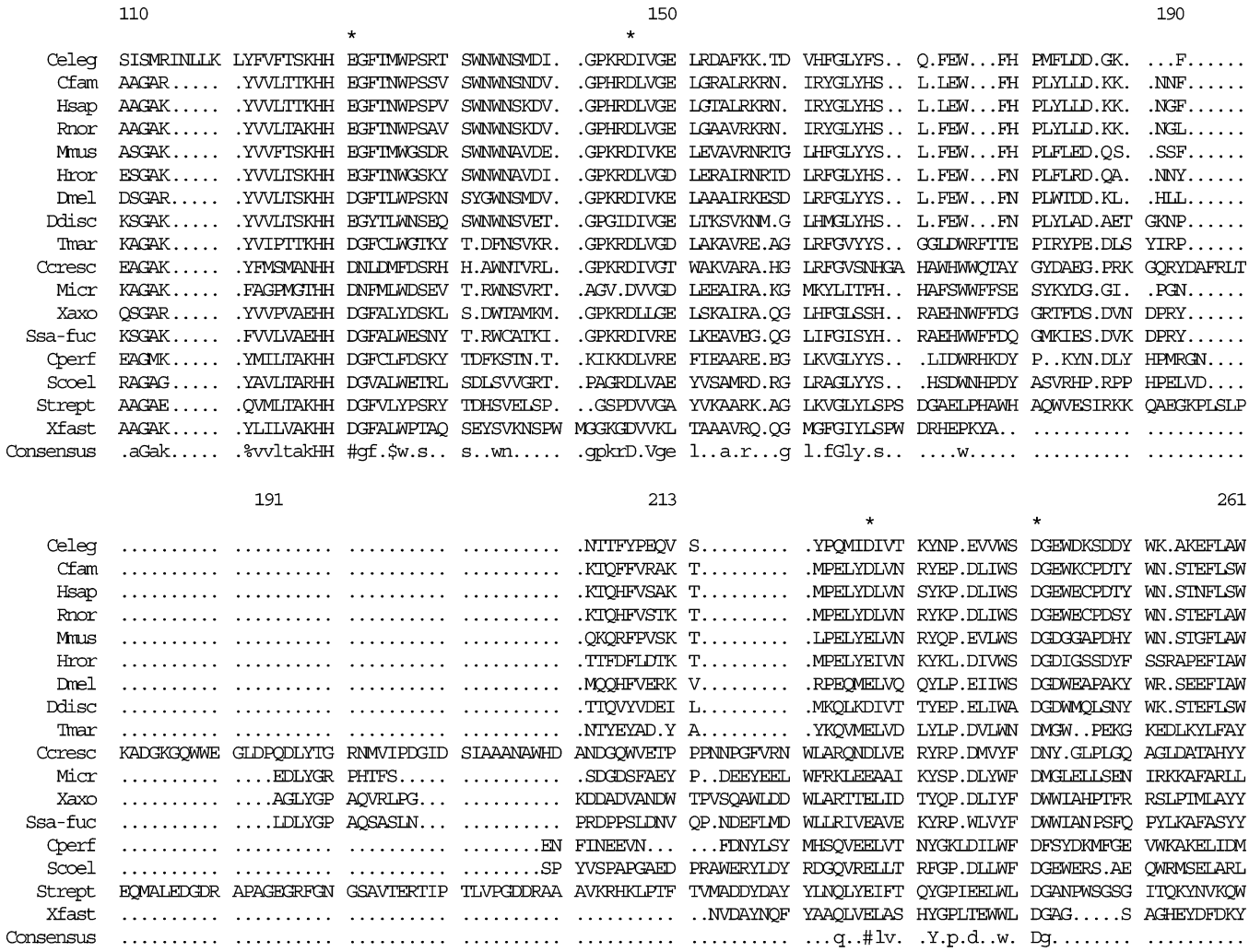


FIGURE 1: Partial multiple sequence alignment of non-redundant amino acid sequences of GH29 α-fucosidases. Sequences were aligned with the program MultAlin (*23*), by following Ssα-fuc numbering. Celeg, *Caenorabditis elegans* (P49713); Cfam, *Canis familiaris* (P48300); Hsap, *Homo sapiens* (Q14334); Rnor, *Rattus norvegicus* (P17164); Mmus, *Mus musculus* (AK002230); Hror, *Halocynthia roretzi* (AB070600); Dmel, *Drosophila melanogaster* (AAF50054.1); Ddisc, *Dictyostelium discoideum* (P10901); Tmar, *Thermotoga maritima* (Q9WYE2); Ccresc, *Caulobacter crescentus* (AAK22780); Micr, *Microscilla* sp. PRE1 (AAK62841.1); Xaxo, *Xanthomonas axonopodis* (AAM37917.1); Ssa-fuc (Ssα-fuc); Cperf, *Clostridium perfringens* (Q8XNK9); Scoel, *Streptomyces coelicolor* (AAD10477); Strept, *Streptomyces* sp. 142 (AAD10477); Xfast, *Xylella fastidiosa* (AAF85750). The amino acids present in low and high consensus (50 and 90%, respectively) are indicated in lowercase and uppercase, respectively. The symbols %, #, and $ indicate any one of F/Y, N/D/Q/E, and L/M amino acids, respectively. The symbol * on the top of the alignment indicates highly conserved D/E.

When assayed in standard conditions at 65 °C, the turnover number (k_{cat}) of the Asp242Gly mutant on pNp−Fuc, 0.24 s^{-1}, results in 1.0×10^{-3} of that of the wild-type activity (245 s^{-1}), suggesting that the mutation affected the active site of the enzyme. The elimination of the catalytic nucleophile typically results in a 10^6−10^8-fold decrease in the catalytic activity of β-glycosidases (*19*). However, higher residual activity in β-glycoside hydrolases mutated in the nucleophile has been observed, generally resulting from wild-type contaminations or translational misincorporation during protein synthesis in *E. coli* (*19*, *24*). For the Asp242Gly mutant, contamination from external sources seems unlikely, because the purification consists of a single glutathione-Sepharose affinity chromatography that is performed with a matrix that is dedicated only to the purification of the mutant. The extent of reactivation in the presence of sodium azide could shed some light on the origin of this residual activity, since limited catalytic acceleration (about 1−2-fold) indicates that the activity could result from tiny wild type contaminations (*19*). To test if the residual activity of Asp242Gly could be increased by external nucleophiles, we assayed the purified mutant on pNp−Fuc at 65 °C in standard conditions with added 2 M sodium azide. At these conditions, the mutant showed a k_{cat} of 11 s^{-1}, indicating a 46-fold activation by azide. As a comparison, the wild-type Ssα-fuc, assayed in 2 M azide, showed a specific activity (339 s^{-1}) comparable to that found at standard conditions (245 s^{-1}). The activation observed in the Asp242Gly mutant by the addition of azide falls in the range typical of β-glycoside hydrolases (10-, 10^7-fold) (*25*, *26*) supporting the hypothesis that the wild-type contamination is negligible.

The effect of external nucleophiles on the activity of the Asp242Gly mutant was further investigated by using different concentrations of sodium azide (Figure 2A). At these conditions, the maximal activity (11 s^{-1}) was found at the highest concentration of nucleophile used. Noticeably, the activity of the mutant was rescued on pNp−Fuc also in the presence of sodium formate. Optimal activity (0.44 U mg^{-1})

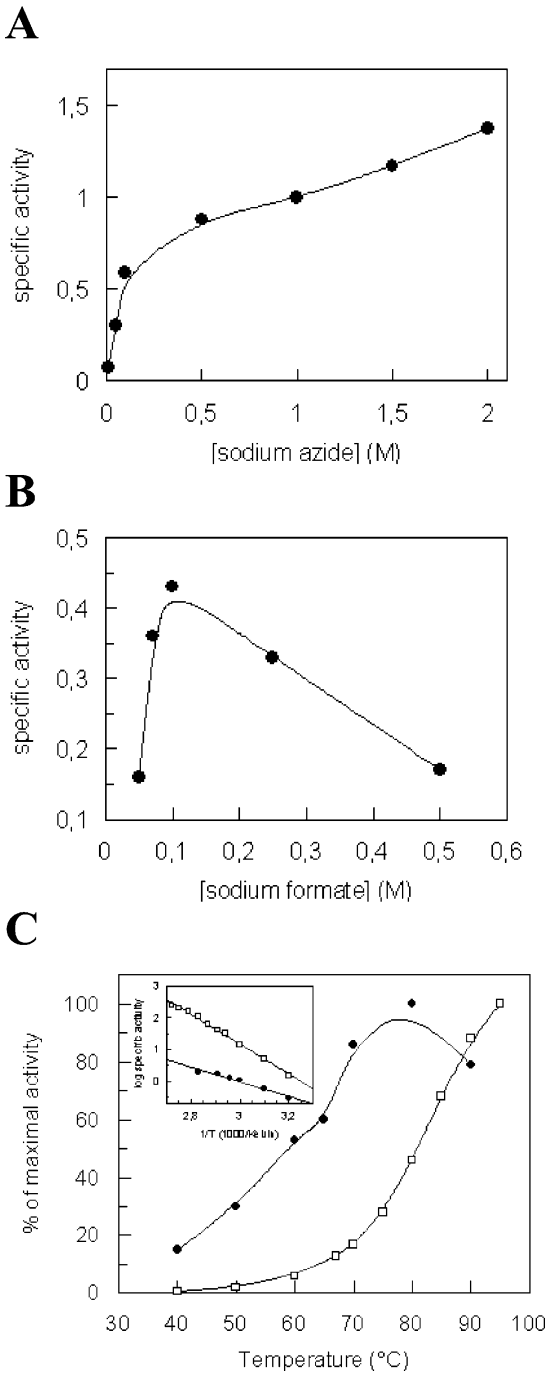


FIGURE 2: Enzymatic characterization of the Asp242Gly mutant. Dependence of the activity on sodium azide (A) and sodium formate (B). Assays were performed at 65 °C on pNp−Fuc as described in Materials and Methods. (C) Dependence on temperature of the wild-type Ssα-fuc (open squares) and Asp242Gly mutant in the presence of 2 M sodium azide (closed circles). The Arrhenius plots (inset) of the mutant and the wild type are reported in the range 40−80 °C and 40−95 °C, respectively.

is observed in 100 mM sodium formate buffer pH 4.0, corresponding to an activation of 16-fold (Figure 2B). Lower activity in 100 mM sodium formate buffer pH 5.0 and 6.0 was found (not shown). In sodium formate buffer pH 4.0, the wild type shows a specific activity of 46 U mg^{-1}, which is only 1.6-fold higher than that found at standard conditions.

The specific activity of the mutant increases with temperature up to 80 °C (Figure 2C) indicating that, after mutation, the enzyme maintains its thermophilicity when reactivated in the presence of azide. The comparison of the energy of activation of the mutant (44 ± 4 kJ mol^{-1}) and the wild type (91 ± 2 kJ mol^{-1}) indicates that the reaction catalyzed by the Asp242Gly mutant depended less on temperature.

Table 1: Steady-State Kinetic Constants of the Wild Type α-Fucosidase[a] and of the Mutant Asp242Gly

	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
wild type	0.028 ± 0.004	287 ± 11	10 250
Asp242Gly	ND[b]	ND	
sodium azide[c]	0.19 ± 0.02	9.66 ± 0.28	51.55
sodium formate[d]	1.03 ± 0.11	5.91 ± 0.20	5.76

[a] Enzymatic assays were performed at 65 °C in 50 mM sodium phosphate buffer pH 6.5. [b] ND (not detectable) means that, using 20 μg of enzyme in the assay, the rates of change in absorbance at substrate concentrations below 0.1 mM did not vary in the experimental conditions and were approximately the same as in the control without enzyme. [c] Assays were performed at 65 °C in 50 mM sodium phosphate buffer pH 6.5, 2 M sodium azide. [d] Assays were performed at 65 °C in 100 mM sodium formate buffer pH 4.0.

The catalytic activity of the Asp242Gly mutant at standard conditions without external nucleophiles was too low for K_M determination at high temperature. Therefore, we measured the steady-state kinetic constants of the mutant on pNp−Fuc in the presence of external nucleophiles at the optimal conditions (Table 1). Sodium azide and sodium formate produced about 0.5 and 0.056% of reactivation of the mutant, respectively, calculated taking as 100% the k_{cat}/K_M of the wild-type enzyme assayed at standard conditions. The higher nucleophilicity of sodium azide, if compared to formate, explains the higher reactivation produced by the former.

The reactivation experiments indicate that the Asp242Gly mutation affected a residue involved in catalysis in Ssα-fuc. To unequivocally determine if the mutation altered the acid/base residue of the reaction or the catalytic nucleophile, we analyzed the stereochemistry of the product of the mutant reactivated by azide. TLC analysis of the reaction mixture containing Asp242Gly, pNp−Fuc, and azide, after incubation at 65 °C, revealed the formation of a new product (R_f = 0.6), distinct from fucose (R_f = 0.4) and pNp−Fuc (R_f = 0.8). This new product was isolated and identified as β-L-fucosyl azide. The identity of the product has been established by an FT-IR spectrum (2119 cm^{-1}) that is identical to that of β-D-fucosyl azide enzymatically synthesized by the β-glycosidase from *S. solfataricus* (data not shown). The anomeric configuration is indicated by the coupling constant (J = 8.1 Hz) of the doublet signal of axial anomeric hydrogen as also observed for β-D-glucosyl azide (*24*), β-D-xylopyranosyl azide (*20*), and authentic β-D-fucosyl azide. The H1−H5 ^{1}H NMR spectrum is reported in Figure 3.

The fact that the fucosyl-azide product obtained by the Asp242Gly mutant is found in the inverted (beta) configuration compared with the substrate (Scheme 2A) allows, for the first time, the unambiguous assignment of Asp242, and its homologous residues, as the nucleophilic catalytic residues of GH29 α-L-fucosidases.

The azide rescue method has been used so far only for β-retaining glycosidase, and it is generally based on substrates that are very reactive (showing leaving groups with $pK_a < 5$) to facilitate the first step of the reaction (Scheme 2). Therefore, it is worth noting that Asp242Gly is efficiently

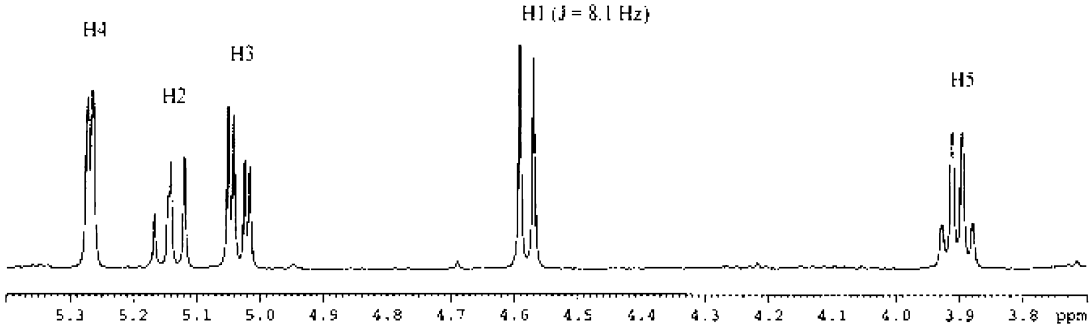


FIGURE 3: H1−H5 ^{1}H NMR spectrum of the fucosyl-azide product. The product was isolated from the reaction of Asp242Gly with pNp−Fuc in the presence of 2 M azide (see Materials and Methods for complete assignment).

reactivated on pNp−Fuc, which shows a leaving group with a pK_a of 7.18. In fact, as a comparison, β-glycosidases from GH1 and GH52, mutated in the nucleophile, could not be reactivated by azide on *p*-nitrophenyl-glycosides, but they require substrates with excellent leaving groups for an efficient chemical rescue of their activity (*19*, *24*). The reactivation of Asp242Gly by azide on pNp−Fuc may be due to the higher specificity constant of Ssα-fuc (10 250 s^{-1} mM^{-1}) if compared to those of the β-glycosidase from *S. solfataricus* (498 s^{-1} mM^{-1}) and of the α-L-arabinofuranosidase/β-D-xylosidase from *Geobacillus stearothermophilus* (130 s^{-1} mM^{-1}) on pNp-β-D-galactoside and pNp-α-L-arabinofuranoside, respectively (*19*, *27*).

We have recently reported that the hydrolytic activity of β-glycosynthases from hyperthermophilic Archaea can be rescued at levels comparable to the corresponding wild-type enzymes in the presence of 50 mM sodium formate buffer pH 3.0 (*28*). This occurs because, in acidic conditions, the acid/base catalyst of these mutated hyperthermophilic β-glycosidases rescued the protonated form performing better the first step of the reaction (*28*). In fact, it has been reported that the charged carboxylic group in the active site, in addition to the nucleophilic attack on the anomeric carbon (Scheme 1), is also responsible for maintaining the correct ionization state of the acid/base residue (*29*). Therefore, the elimination of the nucleophile modifies the ionization state of the acid/base catalyst affecting its catalytic efficiency in the first step of the reaction. We have shown here that the highest reactivation of the Asp242Gly mutant in sodium formate occurs at the lowest pH tested (pH 4.0), strongly indicating that, also in this case, the acidic chemical rescue restored the protonated form of the acid/base residue. The common hyperthermophilic nature of these archaeal enzymes, which resist to the reaction conditions at low pH, allowed a similar chemical rescue of the activity. This confirms that the approach can be of general applicability to α-L- and β-D-glycosidases from hyperthermophiles.

We could not detect the formation of oligosaccharides with the Asp242Gly mutant reactivated in 100 mM sodium formate pH 4.0, suggesting that the enzyme did not act as an α-glycosynthase. This is not surprising; hyperthermophilic β-glycosynthases efficiently produced oligosaccharides by using the substrate *o*-nitrophenyl-β-D-glucoside, in which the *o*-nitrophenol, though showing a pK_a similar to *p*-nitrophenol (pK_a 7.22 and 7.18, respectively), can form a chelate ring by hydrogen bonding increasing the leaving ability upon protonation (*28*). The absence of aryl-α-L-fucosides with groups having better leaving ability hampers the possibility to analyze the reactivation extent of the Asp242Gly mutant with more reactive substrates. Work is in progress to test the α-fucosynthetic potential of this mutant on suitable substrates and the functional role of the invariant residue Asp146 in GH29.

ACKNOWLEDGMENT

We thank Eduardo Pagnotta and Gennaro Scognamiglio for skillful assistance. We thank the TIGEM-IGB DNA sequencing core for the sequencing of the clones.

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BI035036T