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β-Glycosyl Azides as Substrates for α-Glycosynthases: Preparation of Efficient α-L-Fucosynthases

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SUMMARY

Fucose-containing oligosaccharides play a central role in physio-pathological events, and fucosylated oligosaccharides have interesting potential applications in biomedicine. No methods for the large-scale production of oligosaccharides are currently available, but the chemo-enzymatic approach is very promising. Glycosynthases, mutated glycosidases that synthesize oligosaccharides in high yields, have been demonstrated to be an interesting alternative. However, examples of glycosynthases available so far are restricted to a limited number of glycosidases families and to only one *retaining* α-glycosynthase. We show here that new mutants of two α-L-fucosidases are efficient α-L-fucosynthases. The approach shown utilized β-L-fucopyranosyl azide as donor substrate leading to transglycosylation yields up to 91%. This is the first method exploiting a β-glycosyl azide donor for α-glycosynthases; its applicability to the glycosynthetic methodology in a wider perspective is presented.

INTRODUCTION

The great structural variety of carbohydrates results from the diverse stereochemistry of the monosaccharide building blocks and from the enormous number of intersugar linkages that they can form. This feature makes sugar molecules suitable for mediating many biological processes (Varki, 1993), but it complicates greatly their production. In addition, carbohydrate synthesis in vivo, in contrast to nucleic acids and protein synthesis, is a very complex process that is not regulated by universally conserved codes, and has not been automated in vitro yet. This challenge currently motivates the efforts in developing methods for large-scale production of oligosaccharides (Seeberger, 2008). At present, no such methods are available because, in chemical synthesis, most of the difficulties arise from the laborious regio- and stereochemical control. An interesting option is enzymatic synthesis promoted by glycosyltransferases (GT) and glycoside hydrolases (GH) working in transglycosylation mode. The former enzymes are extremely efficient, but their use is hampered by the high cost of the sugar nucleotide substrates, the difficulties in obtaining the catalysts in sufficient amounts, and the extreme specificity of the enzymes (Hancock et al., 2006). Recent successes in improving the promiscuity of GTs by directed evolution have opened new perspectives in the use of these enzymes, but their exploitation is still limited (Aharoni et al., 2006; Williams et al., 2007). An alternative to glycosyltransferases, GHs that retain the anomeric configuration in the product, are widespread enzymes that, using cheap substrates, promote oligosaccharide synthesis by transglycosylation reactions. *Retaining* glycosidases utilize a double displacement mechanism in which a glycosyl intermediate is formed and subsequently hydrolyzed (see Figure S1 available online): when acceptors different from water intercept the glycosyl-enzyme intermediate, transglycosylation reactions occur (McCarter and Withers, 1994). This approach might allow regio- and stereospecificity control, but yields are usually not higher than 40% because the products of the reaction are, at the same time, substrates. To overcome these drawbacks, glycosynthases, a new class of mutant glycosidases derived from *exo*- and *endo*-β-glucosidases by replacing the active site nucleophile with a nonnucleophilic residue, were introduced (Mackenzie et al., 1998; Moracci et al., 1998; Malet and Planas, 1998). The mutation completely inactivates the enzyme, but, in the presence of a substrate with good leaving group ability, the activity of the mutant can be restored. In fact, the small cavity created upon mutation can accommodate a substrate with inverted anomeric configuration when compared with the original substrate (Figure S2A, *inverting* β-glycosynthases) (Mackenzie et al., 1998; Malet and Planas, 1998), or a small anion (Figure S2B, *retaining* β-glycosynthases) (Moracci et al., 1998). In both cases, the mutant enzyme promotes the transglycosylation to an acceptor with almost quantitative yields. *Inverting* β-glycosynthases cannot hydrolyze the product of the reaction showing a β-anomeric configuration, whereas in *retaining* β-glycosynthases the bad leaving ability of the glycosidic group in the products

prevents their subsequent hydrolysis, resulting in quantitative yields.

The approach leading to *inverting* glycosynthases is suitable for both exo- and endo-glycosidases and has successfully been applied to a variety of GHs belonging to several families of the carbohydrate active enzyme classification (Cantarel et al., 2009; http://www.cazy.org/), namely, GH1, GH2, GH5, GH7, GH10, GH16, GH17, GH26, GH31, and GH52. Instead, *retaining* glycosynthases have been obtained only from hyperthermophilic exo-β-glycosidases from family GH1 (for reviews see Perugino et al., 2004, 2005, and Hancock et al., 2006).

In spite of the convenience of this approach, GHs recalcitrant to become glycosynthases are not uncommon (Ducros et al., 2003; Cobucci-Ponzano et al., 2003b; Perugino et al., 2005), which explains why efforts in developing new methods to improve glycosynthases are continuous (Kim et al., 2004; Lin et al., 2004; Ben-David et al., 2008). Recently, glycosynthases have been prepared from enzymes following atypical reaction mechanisms (GH85) and from *inverting* glycosidases (GH8 and GH95) (Umekawa et al., 2008; Honda and Kitaoka, 2006; Honda et al., 2008; Wada et al., 2008). However, these approaches (as stated by the authors), though innovative, do not provide common strategies to convert an *inverting* glycosidase into a glycosynthase (Wada et al., 2008).

The most noticeable examples of enzymes that are not prone to function as glycosynthases are *retaining* α-glycosidases with only a GH31 α-glucosidase available to date (Okuyama et al., 2002). The scarcity of α-glycosynthases has hampered so far the access to the synthesis of a large class of oligosaccharides of biotechnological interest, such as α-L-fucosylated oligosaccharides. Fucose-containing oligosaccharides play a central role in a number of physiological and pathological events (Ma et al., 2006), and therefore fucosylated oligosaccharides have potential applications in biomedicine (Vanhooren and Vandamme, 1999). However, the synthesis of α-L-fucosides by classical chemical methods is challenging because of the remarkable instability of fucosyl donors commonly used in chemical glycosylations, explaining the interest in enzymatic approaches. α-L-Fucosidase-catalyzed reactions led to transfucosylating yields in the range 6%–54% (Murata et al., 1999; Farkas et al., 2000; Wada et al., 2008), whereas mutants obtained by directed evolution of the α-L-fucosidase from *Thermotoga maritima* (Tmα-fuc) increased the transfucosylation yields from 7% (wild-type) to 60% (Osanjo et al., 2007). Noticeably, this result was obtained by mutating three amino acids not directly involved in catalysis.

In the past, we have tried to convert by mutation the *retaining* α-L-fucosidase from the hyperthermophilic archaeon *Sulfolobus solfataricus* (Ssα-fuc) into a novel α-L-fucosynthase under a variety of conditions, but no oligosaccharide product was observed (Cobucci-Ponzano et al., 2008). Here we describe a novel strategy for the production of efficient fucosynthases by using β-L-fucopyranosyl azide as donor substrate. The applicability to the glycosynthetic methodology in a wider perspective is also presented.

RESULTS

Construction of Ssα-fuc Nucleophile Mutants and Kinetic Characterization

The SsD242A and SsD242S mutants of the α-L-fucosidase from *S. solfataricus* were produced as previously described for SsD242G (Cobucci-Ponzano et al., 2003b). As expected, both mutants were almost completely inactive, but the hydrolytic activity of SsD242S was chemically rescued with external nucleophiles resulting in increased activity on both 4-nitrophenyl– and 2-chloro-4-nitrophenyl α-L-fucopyranosides (4NP-α-L-Fuc and 2C4NP-α-L-Fuc) (Figure S3) (for a review on the chemical rescue of the activity of glycoside hydrolase mutants see Ly and Withers, 1999). The increased activity in sodium formate was similar for both substrates (Figure S3A), whereas in sodium azide, SsD242S showed an about 3-fold higher specific activity on 2C4NP-α-L-Fuc if compared with 4NP-α-L-Fuc (Figure S3B). In our hands, SsD242A was not reactivated by sodium azide and was not further characterized.

The steady-state kinetic parameters of the mutants on the 4NP-α-L-Fuc and 2C4NP-α-L-Fuc substrates were measured in several conditions (Table 1); remarkably, the turnover number of SsD242S on 2C4NP-α-L-Fuc in sodium azide (2 M) was 1.8-fold higher than that of the wild-type.

Analysis of the Products of Ssα-fuc Mutants

Incubation of SsD242G and SsD242S in the presence of 4NP-α-L-Fuc or 2C4NP-α-L-Fuc (2 and 20 mM, respectively) and sodium formate did not lead to observable transfucosylation products on thin-layer chromatography (TLC), confirming the results previously reported (Cobucci-Ponzano et al., 2008). Instead, newly formed transfucosylation products were identified in the presence of sodium azide (Figure 1). In the presence of 2C4NP-α-L-Fuc the mutants produced two compounds, which were not UV-visible on TLC. Remarkably, after incubation with the wild-type Ssα-fuc, one of the two compounds was completely hydrolyzed, demonstrating that it contained α-L-anomeric bonds (Figure 1B). The combined use of nuclear magnetic resonance and mass spectrometry allowed us to unequivocally define the structure of products ***1*** and ***2*** (Figure 1C). Compound ***1***, β-L-fucopyranosyl azide (β-L-Fuc-N_3), which is produced with both 4NP- and 2C4NP-α-L-Fuc, is not surprising: SsD242G produced β-L-Fuc-N_3 in similar conditions as expected from a mutant in the nucleophile (Cobucci-Ponzano et al., 2003b). Instead, compound ***2***, the disaccharide α-L-fucopyranosyl–(1-3)-β-L-fucopyranosyl azide (α-L-Fuc-(1-3)-β-L-Fuc-N_3), is unexpected. It is produced only from the 2C4NP-α-L-Fuc and more efficiently by SsD242S (Figure 1B); presumably, the better leaving ability of the 2-chloro-4-nitrophenol aglycon, if compared with the 4-nitrophenol, and the mutation in Ser were decisive to improve the transfucosylation reaction. The presence of the β-L-Fuc-N_3 group at the reducing end of this compound (Figure 1C) indicated that no transfucosylation occurred on 2C4NP-α-L-Fuc, suggesting that it was an efficient donor, but a poor acceptor.

Characterization of the Reaction Mechanism of the Ssα-fuc Mutants

The most surprising result of the sodium azide activity rescue experiments is that mutants in the nucleophile of Ssα-fuc promoted the formation of *both* β-L- and α-L-bonds. Two alternative reaction mechanisms could explain how the Ssα-fuc mutants promoted the synthesis of compound ***2*** containing an α-L-bond. In one case, the β-L-Fuc-N_3 product might become a novel donor and acceptor as a result of its accumulation;

Table 1. Steady-State Kinetic Constants of Wild-Type and Mutant α-L-Fucosidases

	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
Wild-type Ssα-fuc[a]			
4NP-α-L-Fuc	287 ± 11	0.028 ± 0.004	10250
2C4NP-α-L-Fuc	157 ± 9	0.013 ± 0.004	11602
SsD242G			
4NP-α-L-Fuc	0.24[b]	—	—
+ sodium formate pH 4.0			
4NP-α-L-Fuc	5.9 ± 0.2	1.0 ± 0.1	6
2C4NP-α-L-Fuc	1.6 ± 0.1	0.2 ± 0.1	7
+ sodium azide 2 M			
4NP-α-L-Fuc	9.7 ± 0.3	0.19 ± 0.02	51
2C4NP-α-L-Fuc	55 ± 3	0.14 ± 0.02	384
SsD242S			
4NP-α-L-Fuc	0.08[b]	—	—
2C4NP-α-L-Fuc	0.25[b]	—	—
+ sodium formate			
4NP-α-L-Fuc[c]	3.6 ± 0.1	0.9 ± 0.1	4
2C4NP-α-L-Fuc[d]	5.8 ± 0.2	0.045 ± 0.005	129
+ sodium azide 2 M			
4NP-α-L-Fuc	47 ± 1	0.19 ± 0.02	247
2C4NP-α-L-Fuc	286 ± 35	0.3 ± 0.1	987
Wild-type Tmα-fuc[e]			
4NP-α-L-Fuc	80 ± 3	0.033 ± 0.005	2412
2C4NP-α-L-Fuc	88 ± 5	0.007 ± 0.003	12287
TmD224G			
4NP-α-L-Fuc	0.05[b]	—	—
2C4NP-α-L-Fuc	0.46[b]	—	—
+ sodium azide 1 M			
4NP-α-L-Fuc	9.2 ± 0.3	0.015 ± 0.002	613
2C4NP-α-L-Fuc	27 ± 1	0.040 ± 0.005	681

[a] Assays were performed in 50 mM sodium phosphate buffer (pH 6.5) at 65°C.
[b] k_{cat} was determined from the initial velocity at saturating concentration of substrate.
[c] In 1 M sodium formate.
[d] In 2 M sodium formate.
[e] Assays were performed in 50 mM sodium citrate/phosphate buffer (pH 6.0) at 60°C. The standard deviation is reported.

thus, compound ***1*** is transferred to another β-L-Fuc-N_3 molecule leading to α-L-Fuc-(1-3)-β-L-Fuc-N_3 (Figure 2A, lower diagram). This mechanism would imply that azide in β-L-Fuc-N_3 could work as leaving group; though not impossible, this is certainly surprising because glycosyl-azide compounds are rather stable (Ly and Withers, 1999). Therefore, an alternative explanation could be that a third amino acid in the active site of the enzyme might have gained the function of nucleophile as a consequence of the mutation of the Asp242. This hypothetical residue might form the covalent intermediate and then transfer the fucose to the β-L-Fuc-N_3 acceptor (Figure 2B, lower diagram). At the earliest stages of the reaction, following this second hypothesis, sodium azide and the hypothetical nucleophile compete in the attack to the anomeric center of 2C4NP-α-L-Fuc (in Figure 2B compare the upper and lower schemes). To verify this latter hypothesis, we characterized single and double mutants in the residues Glu58 and Glu292, which cooperate with Asp242 in catalysis (Cobucci-Ponzano et al., 2005), but we could not find evidence of their involvement as novel nucleophile. In addition, assuming that a residue acting as nucleophile in SsD242S might react with a mechanism-based inhibitor, we prepared the inhibitor 2-deoxy-2-fluoro-α-L-fucosyl fluoride (2d-2F-Fuc-F, see Supplemental Data), aiming to identify this hypothetical residue by high-performance liquid chromatography (HPLC)/electrospray mass spectrometry as previously reported (Tarling et al., 2003). The mechanism-based inhibitor bound to Asp242 in the wild-type Ssα-fuc used as control whereas the treatment of SsD242S did not produce any labeled peptide (see Supplemental Data). Though these negative results are not conclusive, our data strongly indicate that no additional catalytic nucleophile was produced by mutating Asp242 in Ssα-fuc, making questionable the validity of the reaction mechanism shown in Figure 2B.

To validate the reaction mechanism described in Figure 2A, we tested if SsD242S was able to use β-L-Fuc-N_3 as donor. Remarkably, SsD242S incubated for 16 hr at 65°C in 20 mM β-L-Fuc-N_3 catalyzed the formation of the disaccharide ***2*** with a transfucosylation efficiency, defined as the amount of fucose transferred to an acceptor different from water, measured by HPAEC-PAD, of 40% and exclusive formation of the α-L-(1-3)-bond (Figure 3A). Similar results were obtained with SsD242G, but TLC inspection indicated lower efficiency. β-L-Fuc-N_3 remained unreacted in the absence of the mutant (Figure 3A) or in the presence of the wild-type Ssα-fuc incubated at the same conditions (data not shown), ruling out possible artifacts.

These results confirm the reaction mechanism described in Figure 2A in which the β-L-Fuc-N_3, produced by the mutants from the 2C4NP-α-L-Fuc and azide, become a donor during the course of the reaction.

Construction and Characterization of Tmα-fuc Nucleophile Mutants

The use of the β-L-fucosyl azide donor could be a novel general strategy for the production of efficient fucosynthases, therefore, we prepared mutants in Gly and Ser of the catalytic nucleophile Asp224 of Tmα-fuc, which shows rather low (25%) amino acid sequence identity to Ssα-fuc, but has been fully characterized (Tarling et al., 2003; Sulzenbacher et al., 2004).

As expected, mutations severely affected the fucosidase activity, which could not be rescued in the TmD224S with both sodium azide or sodium formate; thus, this mutant was not further analyzed. Instead, the residual activity of the TmD224G mutant (0.05 and 0.46 s^{-1} on 4NP-α-L-Fuc and 2C4NP-α-L-Fuc, respectively) was rescued only by sodium azide on both substrates (Figure S4). The specific activity was higher on 2C4NP-α-L-Fuc, as reported above for SsD242S (compare Figures S3 and S4).

The steady-state kinetic constants of TmD224G in sodium azide are compared with those of the wild-type in Table 1. The specificity constants of the mutant were 4- and 18-fold lower than those of the wild-type on 4NP-α-L-Fuc and 2C4NP-α-L-Fuc, respectively. However, the affinity for both substrates was

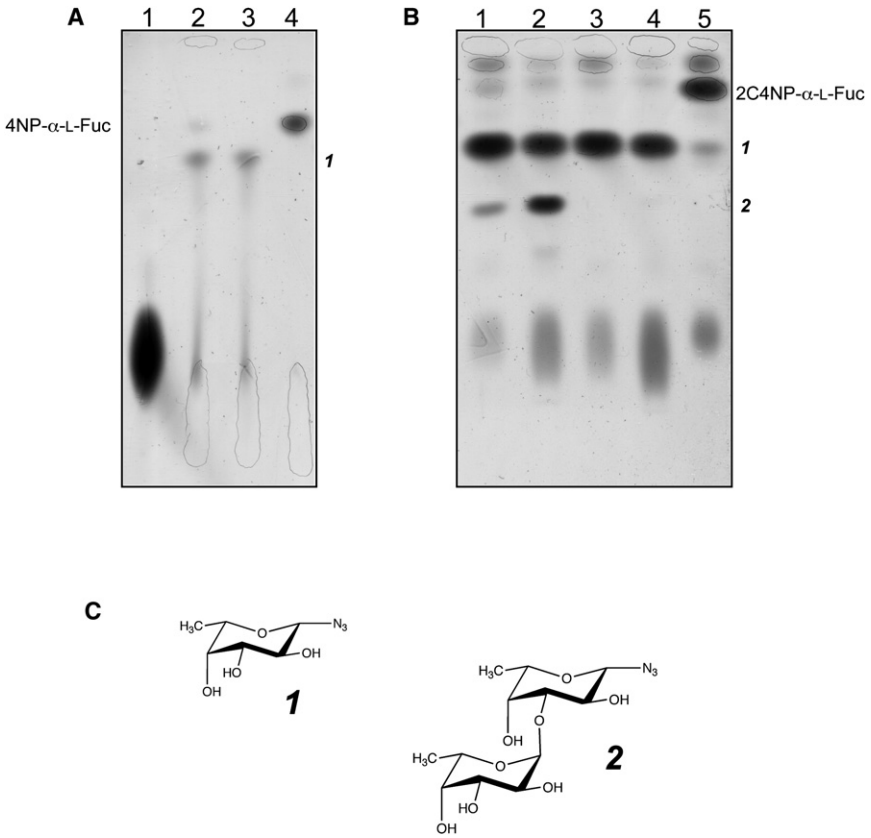


Figure 1. TLC Detection of the Transfucosylation Products of SsD242G/S in Sodium Azide

(A) 2 M sodium azide and 2 mM 4NP-α-L-Fuc; lane 1: fucose marker; lane 2: SsD242G reaction; lane 3: SsD242S reaction; lane 4: blank with no enzyme.

(B) 0.1 M sodium azide and 20 mM 2C4NP-α-L-Fuc: lane 1: SsD242G reaction; lane 2: SsD242S reaction; lanes 3 and 4: the same samples shown in lanes 1 and 2, respectively, after incubation in the presence of wild-type Ssα-fuc; lane 5: blank with no enzyme.

(C) Based on later product analysis, ***1*** and ***2*** turned out to be β-L-Fuc-azide and α-L-Fuc-(1,3)-β-L-Fuc-azide, respectively.

not significantly impaired by the mutation, remaining in the micromolar range.

TLC analysis of the reaction mixtures of TmD224G after incubation at 60°C for 16 hr in sodium azide 0.05–1.0 M and 20 mM 2C4NP-α-L-Fuc revealed a single transfucosylation product of the same polarity of β-L-Fuc-N_3 while no disaccharide products were formed (data not shown). However, incubations in the presence of 10 mM β-L-Fuc-N_3 and 4NP-β-D-Xyl at 1:5 and 1:10 donor:acceptor molar ratios revealed one UV-visible transfucosylation product, showing that TmD224G exploits β-L-Fuc-N_3 for its α-fucosynthase activity (Figure 3B).

Oligosaccharide Synthesis by α-L-Fucosynthases

The substrate specificity of the two α-fucosynthases was analyzed by using β-L-Fuc-N_3 as donor in the presence of glycoside, monosaccharide, or disaccharide acceptors. Remarkably, SsD242S at donor:acceptor molar ratios between 1:2 and 1:3.4, catalyzed the synthesis of products from 16 different acceptors, including aryl-glycosides of hexoses, pentoses, *N*-acetyl-glucosamine, disaccharides, and methylumbelliferyl-fuco- and glucosides (Table S1). In all cases we observed also the formation of the autocondensation product ***2***. Interestingly, SsD242S recognized as acceptors compounds containing either α/β-L/D anomeric bonds.

Also TmD224G in the presence of 10 mM β-L-Fuc-N_3 donor and several acceptors (2- and 4NP-β-D-Xyl, 2NP-β-D-Fuc, and 4NP-β-D-Glc) used at 1:10 donor:acceptor molar ratios revealed several transfucosylation products, but we never observed the autocondensation product (data not shown).

These results showed that the mutants have wide specificity for the acceptor molecule. It is worth noting that most of the donor was converted and all the products accumulated in the reaction after prolonged incubation, as expected for efficient fucosynthases.

To determine the regioselectivity and the ability of SsD242S and TmD224G in synthesizing longer oligosaccharides, we analyzed preparative reactions of the two enzymes. Table 2 summarizes the results of these synthetic trials: reactions I and II show the products of SsD242S in 10 mM of β-L-Fuc-N_3 donor with 4NP-β-D-Xyl and 4NP-β-D-Gal acceptors (34 mM and 20 mM, respectively), whereas in reaction III the mutant was incubated with 5 mM donor and 15 mM 4NP-β-D-GlcNAc acceptor. In reaction IV, TmD224G mutant was incubated with 10 mM β-L-Fuc-N_3 as donor and 100 mM 4NP-β-D-Xyl acceptor.

The products of the reaction were isolated by reverse-phase HPLC and each product was identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, methylation analysis, and ^{1}H-NMR spectroscopy. In the reactions containing SsD242S we always found compound ***2*** and, in trace amounts, α-L-Fuc-N_3 ($[M+Na]^+$ = *m/z* 212.05, NMR: δ_{H1} = 5.37 ppm, $^3J_{H1,H2}$ = 4.3 Hz): this unexpected compound, which is present also in the reaction containing TmD224G, results as trace impurity from the preparation of the donor.

The relative amounts of the different transfucosylation products for each acceptor were obtained from the signal integration of the HPLC chromatogram while the amount of fucose transferred to an acceptor different from water was measured by HPAEC-PAD (as described in Experimental Procedures), yielding the global transfucosylation efficiency (Table 2). When inverted donor:acceptor molar ratios were used, such as 3:1 β-L-Fuc-N_3: 4NP-β-D-Xyl, better total transfucosylation efficiency (76% versus 50%), but also higher amounts of α-L-Fuc-(1-3)-β-L-Fuc-N_3 were found; therefore, an excess of donor was considered detrimental for synthetic purposes.

The regioselectivity of SsD242S depends on the acceptor. The enzyme produced α-(1-3) and α-(1-4) linkages with 4NP-β-D-Xyl,

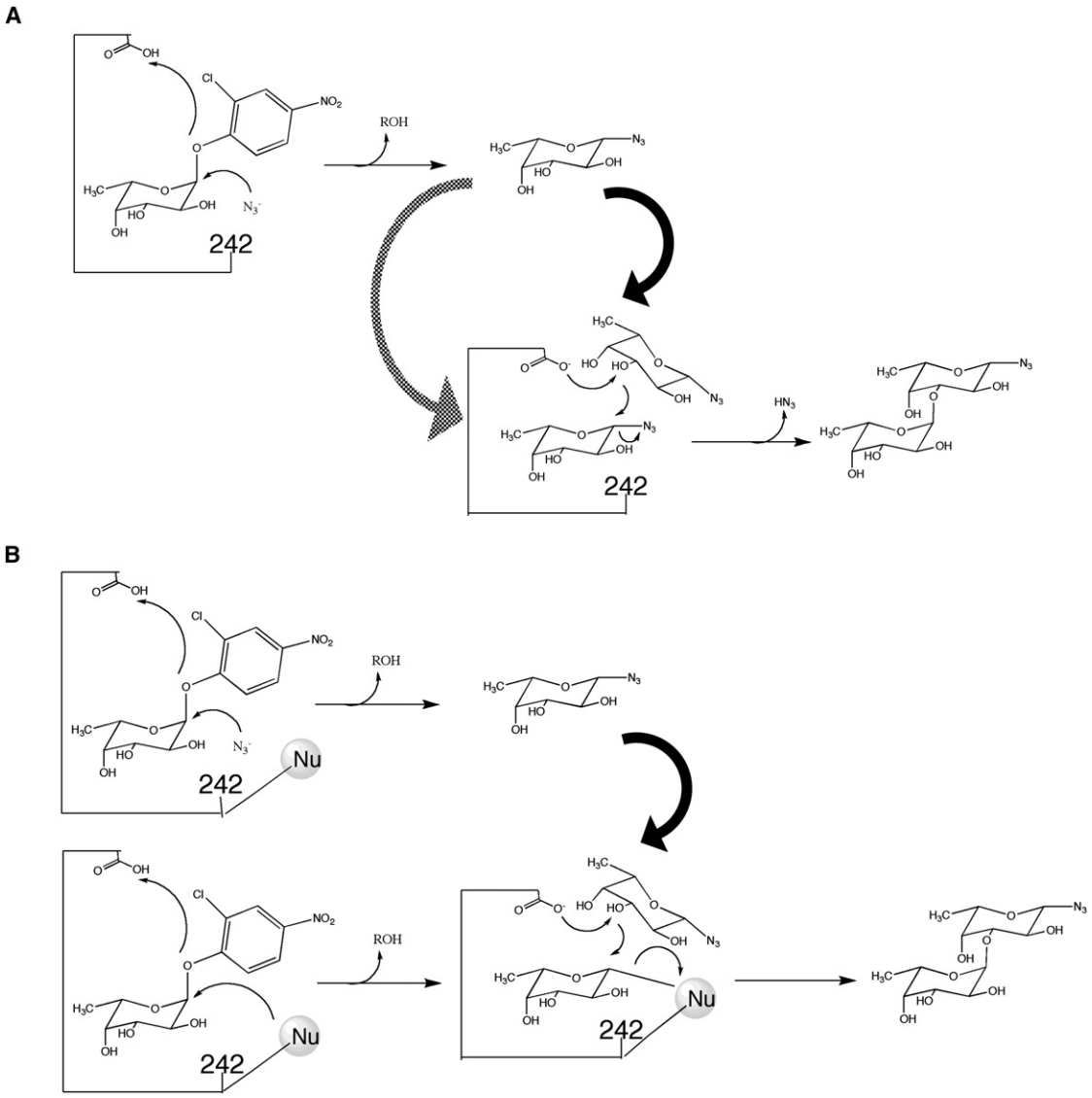


Figure 2. Proposed Reaction Mechanisms of SsD242G/S Mutants in the Presence of 2C4NP-α-L-Fuc and Sodium Azide

The synthesis of α-L-Fuc-(1-3)-β-L-Fuc-N_3 is explained assuming either that azide in β-L-Fuc-N_3 donor works as leaving group leading to self-condensation (A) or with the presence of a putative amino acid in the active site acting as novel nucleophile (B).

whereas with 4NP-β-D-Gal the regioselectivity was switched to the formation of mainly α-(1-6) bond. Instead, with 4NP-β-D-GlcNAc the α-(1-3) regioselectivity prevails with the formation of a single product (***10***). This acceptor-dependent regioselectivity of *exo*-glycosynthases is not novel and has already been described for the *Streptomyces* E384A β-glucosidase (Faijes et al., 2006).

Remarkably, with 4NP-β-D-Xyl and 4NP-β-D-Gal acceptors, the mutant catalyzed the formation of trisaccharides, namely compounds ***5*** and ***9***. The former might be synthesized once compounds ***3*** or ***4*** compete with ***1*** in the acceptor subsite +1 (for the GH active site nomenclature see Davies et al., 1997). Instead, the formation of the trisaccharide ***9***, which showed α-(1-3) and α-(1-2), linkages, might occur only when compound ***8*** acts as acceptor. It is worth noting that compound ***6***, which is the most abundant product isolated in reaction II (78%) and contains an α-(1-6) linkage, did not lead to trisaccharides.

These results suggest that the +1 acceptor binding site of the enzyme has high affinity for compound ***8***; by contrast, the autocondensation product ***2*** is a poor acceptor, as we never observed trisaccharides of fucose. Interestingly, the transfucosylation on disaccharide acceptors always led to branched products (compounds ***5*** and ***9***) and we never observed the transfer to the fucose at the nonreducing end of the acceptor.

The synthetic activity of TmD224G, tested on 4NP-β-D-Xyl acceptor (reaction IV), led to α-(1-4) and α-(1-3) linkage formation in about 1:1 molar ratio; however, this mutant is extremely efficient in transfucosylations (91%).

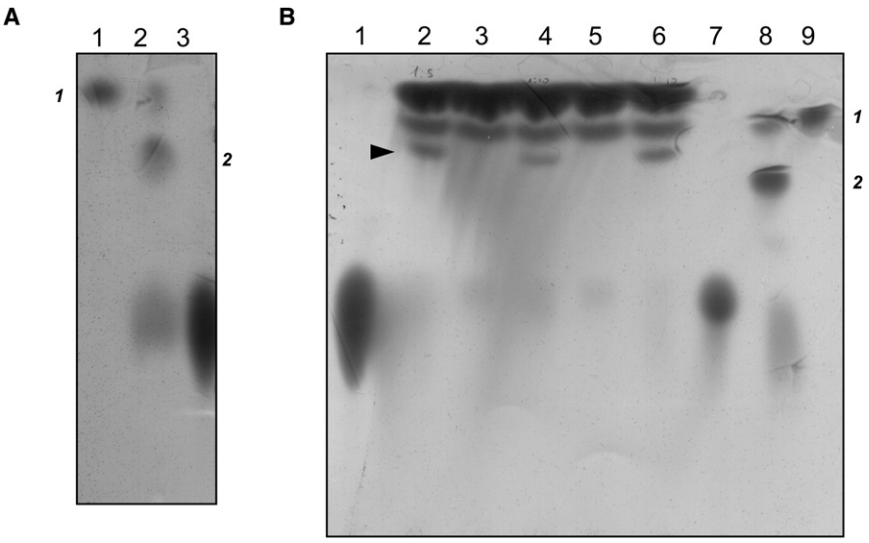


Figure 3. TLC Detection of Transfucosylation Activity of SsD242S and TmD224G with β-L-fuc-azide Donor

(A) Lane 1: blank with no enzyme; lane 2: SsD242S reaction; lane 3: fucose marker.

(B) Lane 1: fucose marker; lane 2: TmD224G (4 μg, 13 pmol) reaction, 1:5 donor:acceptor molar ratio; lane 3 blank with no enzyme; lane 4: TmD224G (1.3 μg, 4 pmol) reaction, 1:10; lane 5 blank; lane 6: as lane 4; lane 7: xylose marker; lanes 8 and 9 are the same as lanes 2 and 1 in (A), respectively. The UV-visible product is indicated by an arrowhead.

Crystal Structure TmD224G in Complex with an Autocondensation Product

Soaking of TmD224G crystals in solutions containing up to 100 mM β-L-Fuc-N_3, followed by diffraction data analysis, did not reveal any electron density for the donor in the active site of the mutant. We thus performed cocrystallization experiments by adding 75 mM β-L-Fuc-N_3 to the crystallization buffer, obtained crystals after several weeks and collected diffraction data to 2.65 Å resolution. Clear electron density could be observed in this case, but, to our surprise, it extended well beyond the donor binding site. Modeling of β-L-Fuc-N_3 into this electron density, in a position equivalent to β-L-fucose in the previously determined crystal structure of the Tmα-fuc product complex (Protein Data Bank code 1odu, Sulzenbacher et al., 2004), and subsequent refinement led to a very clean electron density map around the pyranose ring, but left a substantial amount of residual difference electron density around the azide group. The only reasonable way to clean up the residual electron density and make a good fit to the experimental data was to model a disaccharide into the active site. This observation led us to conclude that prolonged cocrystallization of TmD224G with high concentrations of β-L-Fuc-N_3 yielded an autocondensation product, in contrast to what had been observed in solution. Unfortunately, the modest resolution of the diffraction data did not allow us to identify unambiguously the chemical nature of the autocondensation product. Models containing α-L-Fuc-(1-2/3/4)-β-L-Fuc-N_3 regioisomers gave similar refinement statistics, but the best fit could be obtained with α-L-Fuc-(1-2)-β-L-Fuc-N_3. However, given the limited resolution, the great variety of possible conformational states (chair, boat, skew, etc.) of the pyranose ring at the reducing end could not be accounted for. In the model, which should be taken with caution, the position of the fucose moiety at the nonreducing end and its interactions with surrounding residues are essentially the same as the ones observed in the structure of native Tmα-fuc in complex with L-fucose (Sulzenbacher et al., 2004). The fucosyl moiety at the reducing end makes no interactions with the enzyme, except for a stacking interaction between the azide group and Trp67 (Figure 4). Presumably, reduced interactions might facilitate the departure of the α-L-Fuc-(1-2)-β-L-Fuc-N_3 product from the active site.

It is worth noting that in the crystallization trials we never observed the formation of the complex of TmD224G with β-L-Fuc-N_3. Presumably, the mutation allowed the attack to the anomeric center of the donor by an acceptor leading to the transfucosylation product. Remarkably, in the crystal we observed only the transfucosylation product and not the hydrolytic product (L-fucose), which is efficiently bound by the wild-type enzyme (Sulzenbacher et al., 2004). Possibly, this occurs because Gly224 in the mutant could not form the hydrogen bond to the 1-hydroxyl observed within the wild-type structure in complex with L-fucose (Sulzenbacher et al., 2004).

DISCUSSION

Here we show that new mutants in the catalytic nucleophile of two α-L-fucosidases from the hyperthermophiles *S. solfataricus* and *T. maritima* are efficient α-L-fucosynthases in the presence of activated substrates and sodium azide. This ion usually rescues the enzymatic activity of GH mutants in the nucleophile by acting as external nucleophile and producing glycosyl-azide, which are stable and can be isolated easily and structurally characterized (Ly and Withers, 1999; Zechel and Withers, 2001; Cobucci-Ponzano et al., 2003b; Shallom et al., 2005). In alternative to azide, formate leads to glycosyl-formate intermediates, which, being less stable than the glycosyl-azides, have been exploited for the synthesis of oligosaccharides by *retaining* β-glycosynthases (Moracci et al., 1998; Perugino et al., 2004). Our initial attempts to use this approach on Ssα-fuc mutants failed, possibly because the β-L-fucosyl formate was not stable enough to act as intermediate. In contrast, the chemically rescued activity of SsD242G/S mutants in the presence of sodium azide and 2C4NP-α-L-Fuc substrate led to the synthesis of β-L-Fuc-N_3 (***1***) and, more importantly, of the disaccharide α-L-Fuc-(1-3)-β-L-Fuc-N_3 (***2***), which could also be produced by SsD242S from β-L-Fuc-N_3 donor. Therefore, SsD242G/S mutants can act as α-fucosynthase by following two different mechanisms depending on the reaction conditions: in the presence of sodium azide and 2C4NP-α-L-Fuc substrate, the mutants are able to perform in one pot both the reactions depicted in Figure 2A. Instead, when the mutants are incubated

with suitable acceptors and β-L-Fuc-N_3, they catalyzed transfucosylations (Figure 2A lower part). This is the reaction mechanism followed by *inverting* glycosynthases (Figure S2A); the novelty here is that the donor is fucosyl azide rather than glycoside fluorides in conventional glycosynthases (for a review see Williams and Withers, 2000).

Glycosyl azides are substrates of transglycosylation reactions catalyzed by *retaining* GHs (Fialová et al., 2005); mutants in the catalytic nucleophile can use β-L-Fuc-N_3 because of its stereochemistry and configuration. The higher stability of α-anomers (*axial* bonds) versus β-ones (*equatorial* bonds) is known as the "anomeric effect" (Juaristi and Cuevas, 1992) explaining the much longer half-lives of α-glycosyl fluorides when compared with β-anomers (12 days versus 30 hr for α-D- and β-D-Glc F, respectively [Albert et al., 2000]). In addition, β-L-fucoside derivatives, with $^1C_4$ conformation (compound ***1*** in Figure 2), being 6-deoxyhexopyranosides and showing axial substituents on the C4, are more easily activable than hexopyranosides with C4 equatorial substituents, respectively (Overend, 1972). Therefore, notwithstanding the observation that glycosyl azides are more stable than glycosyl fluorides, β-L-Fuc-N_3 might be reactive enough to function as donor in the transfucosylation reactions catalyzed by the α-fucosynthases, requiring less acid/base assistance.

We have shown here that also the TmD224G mutant can act as α-fucosynthase, confirming that the approach reported is of general application. The regiospecificity and the transfucosylation efficiency differ for the two enzymes and depend on the acceptor used. It is worth noting that the trisaccharide fucosyloligosaccharides (compounds ***5*** and ***9***) and the disaccharide α-L-Fuc-(1-3)-D-GlcNAc (compound ***10***), which is rather common in glycoproteins and in the Lewis^x, Lewis^y, and Sialyl Lewis^x antigens, were easily produced by SsD242S with no efforts to search for optimal reaction conditions. These results suggest that α-L-fucosynthases might be further improved and tailored to specific syntheses.

In the enzymes from *S. solfataricus* and *T. maritima*, only Ser and Gly residues, respectively replacing the natural Asp nucleophile, led to a α-fucosynthase. Ala residues did not work in both enzymes, whereas, intriguingly, Ser, which best acted in the former, did not function in Tmα-fuc. In addition to this, the two enzymes appear also to have different affinities for β-L-Fuc-N_3 in the acceptor binding site. TmD224G forms an autocondensation product only at the high protein and substrate concentrations found in the cocrystallization reaction, suggesting that β-L-Fuc-N_3 is a poor acceptor. Unfortunately, no electron density could be observed beyond subsite +1, precluding further analysis. Likewise, presuming disorder due to high mobility, no electron density could be observed in the region succeeding the acid/base Glu266, apparently important for transfucosylation reactions. Indeed, a directed evolution study of Tmα-fuc reported that mutations Thr264Ala and Tyr267Phe enhance considerably the transglycosylation activity of the enzyme (Osanjo et al., 2007). Structural data show that the side chain of Thr264 establishes a strong hydrogen bond with the side chain of Arg254, whereas residues Ala265-His268 do not make contact with any other residue of the protein, suggesting high mobility in this region (Sulzenbacher et al., 2004). Abolishing the anchoring of Thr264 to Arg254 by a Thr264Ala mutation presumably increases the mobility even more, possibly reorienting amino acids to make direct contact with the substrate and increasing the transfucosylation activity.

When we compare the crystal structure of Tmα-fuc with a homology model of Ssα-fuc, we observe striking differences in the acceptor binding site. Tmα-fuc residues Thr264 and Arg254 are replaced by Ile287 and Val273, respectively: the side chain of Ile287 makes only weak hydrophobic contact with a nearby methionine, and consequently the loop region carrying the Ssα-fuc acid/base Glu292 appears to be even less anchored to the rest of the protein than in Tmα-fuc. In subsite +1 Tmα-fuc Met225 is replaced in Ssα-fuc by Trp234, and this latter residue might provide a better stacking platform for pyranoside acceptors. Furthermore, a long insertion, missing in Ssα-fuc, is found in Tmα-fuc after β strand β1, narrowing the acceptor binding site. Residues found in this insertion are likely to impose a steric and polar stringency on the nature of the acceptor sugar, not encountered in more open binding sites. These observations indicate that minor structural changes in the donor and acceptor binding sites dictate the glycosynthase activity. Therefore, exploration of different strategies in order to identify the best donor/acceptor substrates in a particular catalytic context is necessary.

SIGNIFICANCE

We report here the preparation of two novel *retaining* α-fucosynthases, which follow the classical reaction pathway proposed more than 10 years ago for β-glycosynthases (Mackenzie et al., 1998), but utilize β-fucosyl azide as donor substrate. Our findings might open new perspectives in the use of azide derivatives for the production of novel α-glycosynthases. The only α-glucosynthase known so far utilizes as donor the β-Glc-F (Okuyama et al., 2002), which has a half-life of 30 hr in several aqueous buffer systems. Moreover, other glycoside fluoride derivatives are even less stable: β-D-galactopyranoside- and β-D-mannopyranoside fluorides have half-lives ranging between 6 and 8 hr and 11 and 17 hr, respectively (Albert et al., 2000). Therefore, fluorinated substrates in enzymatic α-galactosynthetic and α-mannosynthetic reactions, often taking long incubations, might be inappropriate substrates due to their enhanced spontaneous hydrolysis at the operational conditions. In these regards, azide derivatives might show the right balance between stability and reactivity to work as suitable donors in α-glycosynthetic reactions. Future work in the development of novel glycosynthases should take into account the relative stability of donors derivatized with different chemical groups.

EXPERIMENTAL PROCEDURES

Chemicals

All commercially available substrates were purchased from Sigma-Aldrich. The β-L-Fuc-N_3 was chemically synthesized from L-Fuc in three steps and 67% yield according to a published procedure (Kunz et al., 1991). The GeneTailor Site-directed Mutagenesis System was from Invitrogen; the synthetic oligonucleotides (Table S2) were from PRIMM (Italy), and the His_6 tagged proteins were purified with the Protino Ni-TED 1000 protein purification system (Macherey-Nagel, Germany).

Table 2. Synthetic Products of SsD242S and TmD224G

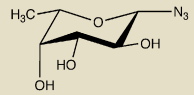
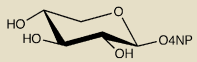
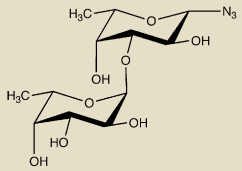
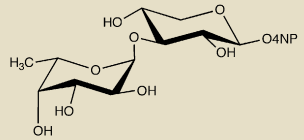
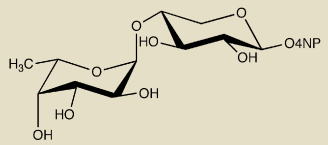
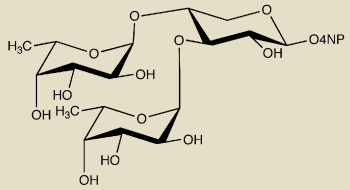
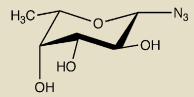
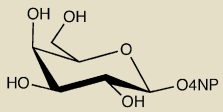
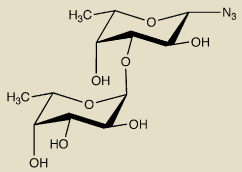
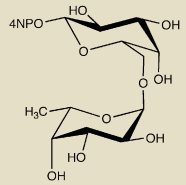
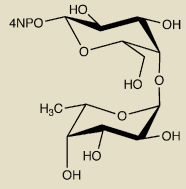
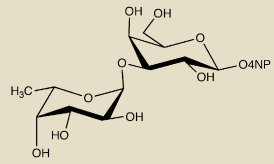
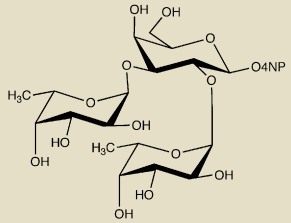
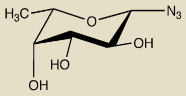
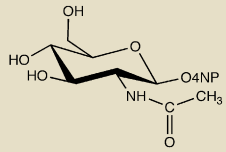
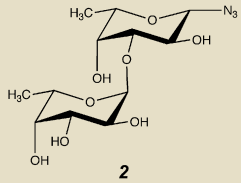
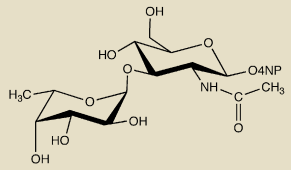
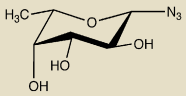
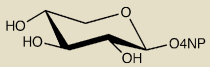
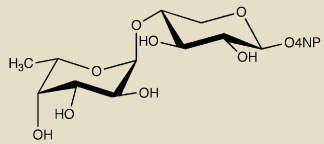
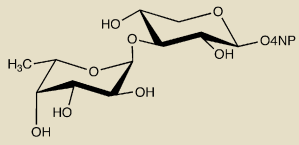
Reaction	Donor	Acceptor	Products	Relative Ratios of Transfucosylation Products (%)
I			 ***2***	ND[a]
			 ***3***	53
			 ***4***	31
			 ***5*** Total transfucosylation efficiency 50%	16
II			 ***2***	ND
			 ***6***	78
			 ***7***	10

Table 2. *Continued*

Reaction	Donor	Acceptor	Products	Relative Ratios of Transfucosylation Products (%)
			 ***8***	8
			 ***9*** Total transfucosylation efficiency 26%	4
III			 ***2***	ND
			 ***10*** Total transfucosylation efficiency 86%	100
IV			 ***4***	55
			 ***3*** Total transfucosylation efficiency 91%	45

[a] ND, not determined.

Site-Directed Mutagenesis

The plasmid pGEX-frameFuc expressing Ssα-fuc and the preparation of SsD242G were described previously (Cobucci-Ponzano et al., 2003a, 2003b). Mutants SsD242A/S were prepared by site-directed mutagenesis from the pGEX-frameFuc plasmid. Double mutants SsD242G/E58G-E292G were prepared by polymerase chain reaction by using as template the vector

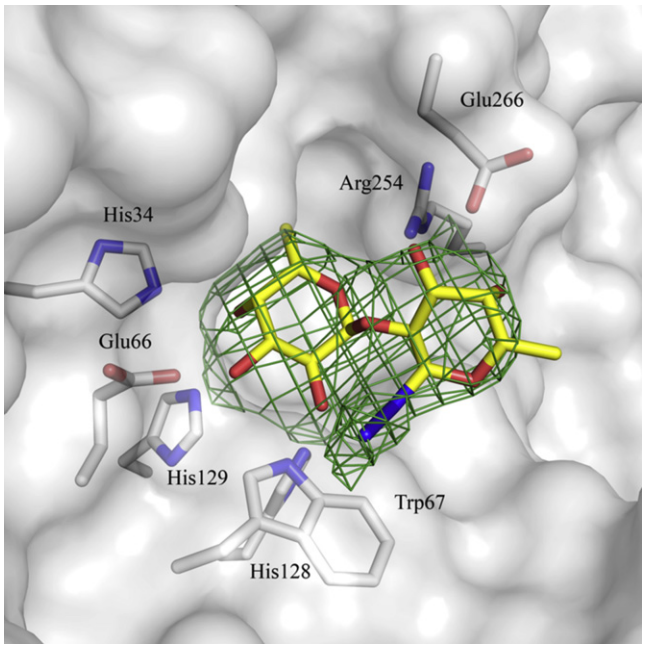


Figure 4. Close-up View of the Active Site of TmD224G
α-L-Fuc-(1-2)-β-L-Fuc-N_3 is shown in stick representation with carbon, oxygen, and nitrogen atoms colored in yellow, red, and blue, respectively. Residues interacting with the disaccharide are shown as sticks under a transparent surface, with carbon, oxygen, and nitrogen atoms colored in gray, red, and blue, respectively. A maximum-likelihood/σA-weighted Fo-Fc electron density calculated prior incorporation of the disaccharide into the model and contoured at 2.5 σ is shown in green.

expressing SsD242G and the mutagenic oligonucleotides reported previously (Cobucci-Ponzano et al., 2005).

The plasmid expressing Tmα-fuc was described previously (Sulzenbacher et al., 2004) and it was used as template for the preparation by site-directed mutagenesis of TmD224G and TmD224S. The genes containing the desired mutations were identified by direct sequencing and completely resequenced.

Expression and Purification of Mutant α-L-Fucosidases

The mutant α-L-fucosidases from *S. solfataricus* were expressed as previously described and purified by a slight modification of the final heating steps at 65°C and 70°C (Cobucci-Ponzano et al., 2003b). The mutants of the *T. maritima* α-L-fucosidases were expressed as previously reported (Tarling et al., 2003). The purification procedures were performed with affinity matrixes dedicated only to the purification of the specific mutant to exclude contamination by the wild-type enzyme from external sources. The enzymes, > 95% pure by SDS-PAGE, stored at 4°C in sodium phosphate buffer 20 mM (pH 7.0), NaCl 150 mM (phosphate-buffered saline) were stable for several months at 4°C. Protein concentration was determined with the method of Bradford (Bradford, 1976), by using bovine serum albumin as standard.

Enzymatic Characterization

The activity of the wild-type and mutant *Ssα-fuc* at standard conditions was measured at 65°C in 50 mM sodium phosphate buffer (pH 6.5, buffer A), with 4NP-α-L-Fuc (1 mM) and 2C4NP-α-L-Fuc (3 mM) by using up to 20 μg (40 pmol) enzyme. The activity of TmD224G was measured on the same substrates and concentrations at 60°C in 50 mM sodium citrate/phosphate buffer (pH 6.0, buffer B).

The chemically rescued activities of SsD242S and TmD242G on the same substrates, in the presence of sodium formate or sodium azide, were measured at the indicated conditions.

The molar extinction coefficients used are reported in Table S3. For all these enzymes, one unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol substrate in 1 min at the conditions described.

Steady-state kinetic parameters of *Ssα-fuc* and SsD242G/A/S mutants on 4NP-α-L-Fuc and 2C4NP-α-L-Fuc were described previously (Cobucci-Ponzano et al., 2003b, 2008). Steady-state kinetic parameters of wild-type *Tmα-fuc* and TmD224G were measured in buffer B at 60°C on 4NP-α-L-Fuc and 2C4NP-α-L-Fuc used at concentrations ranges of 0.005–0.8 mM, and, where indicated, in the presence of 1 M sodium azide. The amount of the enzyme used in the assays was 5 μg (16 pmol).

In all the enzymatic assays, spontaneous hydrolysis of the substrate was subtracted by using appropriate blank mixtures without enzyme. All kinetic data were calculated as the average of at least two experiments and were plotted and refined with the program *GraFit* (Leatherbarrow, 1992).

Glycosynthetic Trials

To purify and characterize the glycosynthetic products, SsD242G (50 μg, 100 pmol) was incubated for 16 hr at 65°C in 0.8 ml buffer A in the presence of 0.1 M sodium azide, and 20 mM 2C4NP-α-L-Fuc. The transfucosylation products of SsD242S from β-L-Fuc-N_3 donor to different acceptors were prepared by incubating 94 μg (188 pmol) enzyme for 16 hr at 65°C in 0.8 ml buffer A, by using different donor:acceptor molar ratios. The transfucosylation products of TmD224G were prepared by incubating 38 μg (122 pmol) enzyme for 16 hr at 70°C in 0.2 ml buffer A by using 10 mM β-L-Fuc-N_3 donor and 100 mM 4NP-β-D-Xyl (1:10 molar ratio). Blank mixtures without enzyme were also prepared. The products were separated on a silica gel 60 F_{254} TLC using ethyl acetate-methanol-water (70:20:10) as eluent and were detected by exposure to 4% α-naphthol in 10% sulfuric acid in ethanol followed by charring.

The reaction mixtures were separately frozen dried and the purification of each sample was obtained by reverse-phase chromatography (Polar-RP 80A, Phenomenex, 4 μ, 250 × 10 mm) on an Agilent HPLC instrument 1100 series, using 3:2 water/methanol as eluent. The eluted products were first analyzed by positive ions reflection MALDI-TOF mass spectrometry. For characterization of the products, see Supplemental Experimental Procedures.

Analysis of transfucosylation efficiency of Ssα-fuc and Tmα-fuc mutants, was performed by use of a high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) equipped with a PA1 column (Dionex, USA). The reaction mixtures and the blank mixtures described above were diluted (10- to 100-fold) with H_2O and 0.5 nmol arabinose as internal standard, loaded onto the PA1 column and eluted with 16 mM NaOH. The moles of fucose were determined by integration of the peaks within the chromatogram, based on fucose and arabinose standard curves. The amount of fucose transferred by the enzyme to water was calculated by subtracting the amount of free fucose measured in the blank mixtures from that identified in the reaction mixtures.

To measure the total amount of fucose enzymatically transferred, 1/10 of the reaction mixtures were incubated for 90 min at 65°C in the presence of 1.2 μg (2.4 pmol) Ssα-fuc wild-type. Successively, the solution was treated as described above and run by HPAEC-PAD to measure the total amount of fucose. The efficiency of the transfucosylation reaction was calculated as: total amount of fucose transferred − moles of fucose transferred to water / total amount of fucose transferred × 100.

Crystallographic Analysis

Crystals of TmD224G in complex with β-L-Fuc-N_3 were obtained as reported for the native enzyme (Sulzenbacher et al., 2004), with the addition of 14 mg/ml β-L-Fuc-N_3 to the crystallization solution. Crystals belong to space group H32 and contain two molecules of TmD224G in the asymmetric unit. Diffraction data extending to 2.65 Å were collected from a flash-frozen crystal at the ESRF beam line ID14-2. Data were indexed and integrated with MOSFLM (Leslie, 1992) and all further computing was carried out with the CCP4 program suite (CCPN, 1994), unless otherwise stated. Data collection statistics are summarized in Table 3. The structure of TmD224G in complex with β-L-Fuc-N_3 was solved by molecular replacement with the program PHASER (McCoy et al., 2007), using the crystal structure of native Tmα-fuc (Protein Data Bank code 1hl8) as a search model, and the resulting model was refined with REFMAC (Murshudov et al., 1997) using the maximum likelihood approach and incorporating bulk solvent corrections and anisotropic

Table 3. Crystallographic Data Collection and Refinement Statistics

Data	
Wavelength	0.933
a = b, c (Å)	180.25, 169.73
Resolution range[a]	35 – 2.65 (2.79 – 2.65)
R_{merge}[a,b]	0.080 (0.455)
No. of observations	207721
No. of unique reflections	30789
Completeness (%)[a]	99.9 (100.0)
Redundancy[a]	6.7 (6.5)
$\langle I/\sigma I \rangle$[a]	16.5 (3.8)
B from Wilson statistics	65.3
Refinement	
Resolution (Å)	35-2.65
No. of protein atoms[c]	7168
No. of water molecules/ligand atoms[c]	103 / 46
R_{cryst}[d] / R_{free} (%)	20.14 / 24.06
Rmsd 1-2 bond distances (Å)	0.006 (0.020)
Rmsd 1-3 bond angles (°)	0.949 (1.95)
Average main/side chain B (Å^2)	52.15 / 53.07
Average B solvent / ligand (Å^2)	24.37 / 60.26
Main chain / side chain Δ B, bonded atoms (Å^2)	0.77 / 1.14

[a] Values in parentheses are for the highest resolution shell.
[b] $R_{merge} = \Sigma_{hkl} \Sigma_i | I_{hkli} - \langle I_{hkli} \rangle | / \Sigma_{hkl} \Sigma_i \langle I_{hkli} \rangle$.
[c] Per asymmetric unit, corresponding to 2 molecules of TmD224G. Target mean values and standard deviations are given in parentheses.
[d] $R_{cryst} = \Sigma ||F_o| - |F_c|| / \Sigma |F_o|$.

F_{obs} *versus* F_{calc} scaling. A random 5% (1536) of reflections were set aside for cross-validation purposes. Manual adjustments of the model were carried out with Coot (Emsley and Cowtan, 2004). Refinement and structure quality statistics are listed in Table 3. Coordinates have been deposited in the Protein Data Bank (Berman et al., 2000) with accession reference number 2wsp. Figure 4 was generated using the program PYMOL (http://pymol.sourceforge.net/) (DeLano, 2002). A homology model of Ssα-fuc was generated with the Phyre Server (http://www.sbg.bio.ic.ac.uk/phyre/) (Kelley and Sternberg, 2009).

ACCESSION NUMBERS

Coordinates have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank with the accession code 1odu.

SUPPLEMENTAL DATA

Supplemental Data include four figures, three tables, and Supplemental Experimental Procedures, and can be found with the article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00322-6.

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