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Activity of Hyperthermophilic Glycosynthases Is Significantly Enhanced at Acidic pH†

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ABSTRACT: We have previously shown that the hyperthermophilic glycosynthase from *Sulfolobus solfataricus* (Ssβ-glyE387G) can promote the synthesis of branched oligosaccharides from activated β-glycosides, at pH 6.5, in the presence of 2 M sodium formate as an external nucleophile. In an effort to increase the synthetic potential of hyperthermophilic glycosynthases, we report a new method to reactivate the Ssβ-glyE387G glycosynthase and two novel mutants in the nucleophile of the β-glycosidases from the hyperthermophilic Archaea *Thermosphaera aggregans* (Taβ-gly) and *Pyrococcus furiosus* (CelB). We describe here that, at pH 3.0 and low concentrations of sodium formate buffer, the three hyperthermophilic glycosynthases show k_{cat} values similar to those of the wild-type enzymes and 17-fold higher than those observed at the usual reactivation conditions in 2 M sodium formate at pH 6.5. Moreover, at acidic pH the three reactivated mutants have wide substrate specificity and improved efficiency in the synthetic reaction. The data reported suggest that the reactivation conditions modify the ionization state of the residue acting as an acid/base catalyst. This new reactivation method can be of general applicability on hyperthermophilic glycosynthases whose intrinsic stability allows their exploitation as synthetic tools at low pH.

The enzymatic synthesis of oligosaccharides, which play a variety of functions in living organisms (*1*, *2*), represents a powerful alternative to the classical chemical methods. The use of glycosyltransferases, despite their high regioselectivity, is hampered by their scarce availability and stability and by the high cost of the nucleoside phosphate donors (*3*, *4*). An alternative approach is the use of glycoside hydrolases by reverse hydrolysis (equilibrium-controlled synthesis) or transglycosylation (kinetically controlled process). Transglycosylation is performed by retaining glycosidases that contain two carboxylic acid residues in the active site, one acting as a nucleophile, the other as an acid/base catalyst, with the reaction proceeding via a covalent glycosyl-enzyme intermediate (*5*−*7*) (Scheme 1A).

Transglycosylation occurs when the glycone of the glycosyl-enzyme intermediate is transferred to an acceptor rather than to water. The major drawback of this approach is that the reaction products are themselves targets of the hydrolytic activity of the glycosidase, thus reducing the yields of the process. Recently, the advent of glycosynthases, retaining glycoside hydrolases mutated in the catalytic nucleophile, gave the opportunity to synthesize complex oligosaccharides in high yields (*8*). Typically, mesophilic glycosynthases employ, as donors, glycosyl-fluoride substrates that, possessing the opposite anomeric configuration to that of the normal substrate, mimic the glycosyl-enzyme intermediate. Therefore, β-retaining glycosynthases catalyze the synthesis of the products with a mechanism similar to the second step of the retaining hydrolysis reaction, in which the carboxylate of the active site acts as a general base, promoting the attack of the acceptor to the α-glycosyl-fluoride (Scheme 1B) (*8*−*10*).

Instead, the glycosynthase from *Sulfolobus solfataricus* (Ssβ-glyE387G),[1] the only hyperthermophilic enzyme of this class available so far, can promote the synthesis of branched oligosaccharides from activated aryl-β-glycosides in the presence of an external nucleophile (i.e., sodium formate) by following the mechanism described in Scheme 1C (*11*, *12*).

In the first step of the reaction, the external nucleophile finds room in the active site of the enzyme depleted of the catalytic nucleophile and attacks the anomeric center of the substrate; in this way, the acid/base catalyst promotes the

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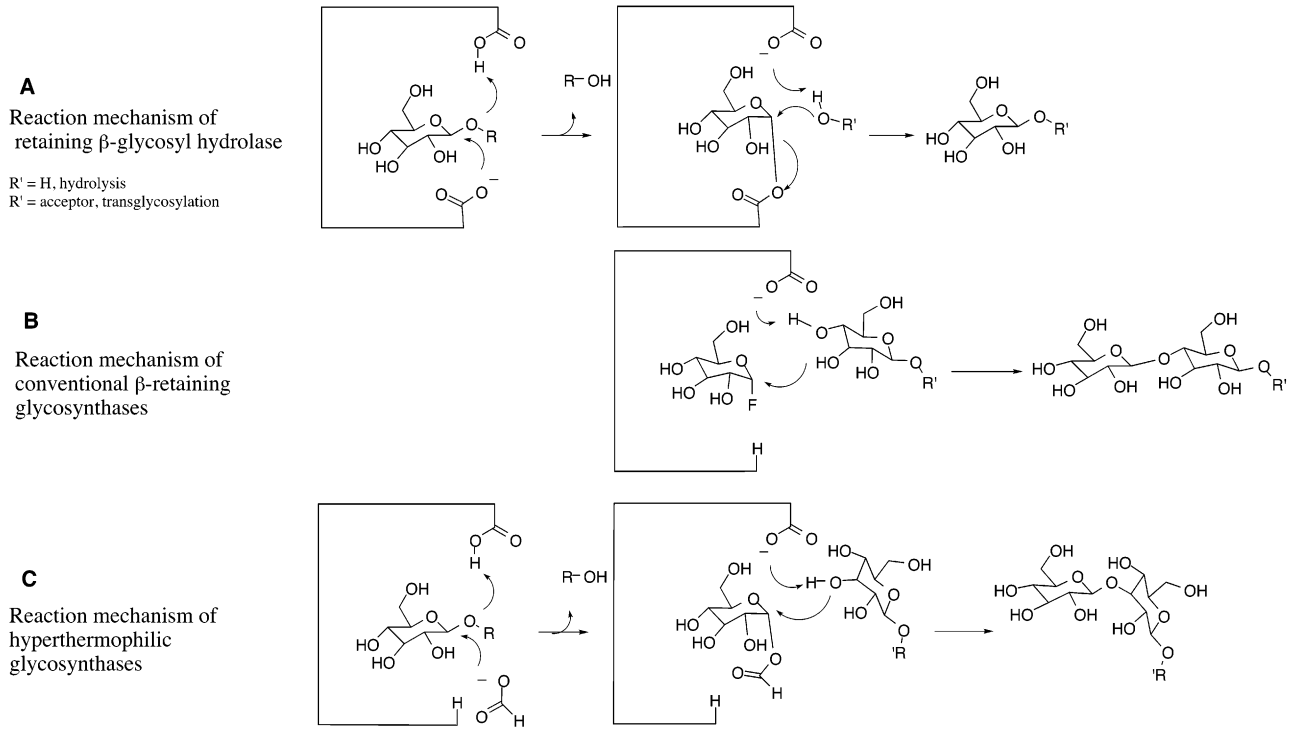
Scheme 1



departure of the aglycon group of the substrate, whereas sodium formate and the glyconic group form a metastable intermediate. In the second step, the attack of the intermediate by an acceptor molecule, together with the action as a general base of the carboxylate, completes the reaction allowing the formation of the product. The oligosaccharides produced by the mechanisms shown in Scheme 1B,C accumulate in the reaction mixture because they are not activated donors and cannot be hydrolyzed by the glycosynthases.

It is evident from Scheme 1C that the leaving ability of the group in the substrate, and the protonated form of the carboxylic group in the enzyme, are essential to perform efficiently the first step of the reaction. Evidence has been provided that the removal of the catalytic nucleophile in retaining glycosidases causes a downward shift in the pK_a of the acid/base catalyst (*13*). As a consequence, this group, at neutral pH, is converted to the ionized form, and it may perform the first step of the reaction less efficiently.

To take advantage of the extreme stability of hyperthermophilic enzymes in harsh conditions, we decided to analyze the activity of hyperthermophilic glycosynthases in acidic conditions. We assume that below pH 6.0 the acid/base catalyst would be converted to the protonated form necessary to complete efficiently the first step of the glycosynthetic reaction (Scheme 1C). To this aim, we have analyzed the glycosynthetic activity in 50 mM sodium formate buffer pH 3.0−6.0 of the Ssβ-glyE387G mutant and of two other mutated β-glycosidases from the hyperthermophilic Archaea *Thermosphaera aggregans* (Taβ-gly) and *Pyrococcus furiosus* (CelB) (*14*, *15*).

We report here that the three hyperthermophilic glycosynthases produced 2-Np-oligosaccharides from 2-Np-Glc. The mutants, which showed their activity restored at wild-type levels in diluted formate buffer at a pH below 5.0, displayed different substrate specificity forming products with different degrees of polymerization. We propose that the ionization state of the acid/base catalyst in the active site at these conditions is crucial for the high reactivation observed. These studies show that hyperthermophilic glycosynthases could represent a novel tool for the enzymatic synthesis of oligosaccharides.

MATERIALS AND METHODS

Reagents. All commercially available substrates were purchased from Sigma. *Pfu* DNA polymerase was from Stratagene, and deoxynucleoside triphosphates were from Roche Molecular Biochemicals. Restriction endonucleases and T4 DNA ligase were from New England BioLabs. The pET9d expression vector was from Novagen. Synthetic oligonucleotides were from Genenco (Italy).

Plasmid Preparation. The plamids expressing the β-glycosidases from *S. solfataricus* (Ssβ-gly) and *P. furiosus* (CelB) were described previously (*11*, *16*). The gene encoding the β-glycosidase from *T. aggregans* (Taβ-gly) was amplified by a polymerase chain reaction (PCR) from the genomic DNA of *T. aggregans* prepared by using the NucleoSpin Tissue kit (Mackerey-Nagel, Germany). The synthetic oligonucleotides used were the following: 5′-GCGCGCCATGGCAAAATTCCCCAAAGACTTCATGATA-3′ and 5′-CGCGCGGATCCTTCAGGATCAGTGTAAGATG-3′.

[1] Abbreviations: 1 Ssβ-gly, *Sulfolobus solfataricus* β-glycosidase; CelB, *Pyrococcus furiosus* β-glycosidase; Taβ-gly, *Thermosphaera aggregans* β-glycosidase; 2-Np-Glc, 2-nitrophenyl-β-D-glucopyranoside; 2,4-DNp-Glc, 2,4-dinitrophenyl-β-D-glucopyranoside; 4-Np-Glc, 4-nitrophenyl-β-D-glucopyranoside; 2,4-DNp-2F-β-Glc, 2,4-dinitrophenyl-2-deoxy-2-fluoro-β-glucoside; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; ORF, open reading frame; FPLC, fast protein liquid chromatography; ϵ_M, molar extinction coefficient; GOD-POD, glucose oxidase-peroxidase enzymatic assay; NMR, nuclear magnetic resonance; GST, glutathione *S*-transferase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; OD, optical density; TLC, thin-layer chromatography; COSY, correlation spectroscopy; FAB-MS, fast atom bombardment-mass spectrometry.

These primers, which introduce a NcoI and a BamHI site (underlined) at the 5′, just before the first ATG, and 3′ ends of the open reading frame (ORF), respectively, were designed on the basis of the sequence available in the data bank (Q9YGA8). The insertion of the NcoI site determined the creation of an additional codon GCA (encoding an Ala residue) after the first ATG of the protein. The resulting DNA fragment was cloned in pET9d expression plasmid, in which Taβ-gly is under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase promoter that drives high expression levels in bacterial hosts (*17*). After ligase reaction, three independent and identical clones were identified by DNA sequencing, and they turned out to be different from the gene Q9YGA8. In particular, they carried the mutations N62Y, Q63K, L231F, V303A, and A412V; therefore, the gene was cloned again as described above, but all the clones obtained showed the same mutations. As a further control, the Taβ-gly gene was amplified as described above, and the PCR product was directly sequenced; again, the same mutations were found with no ambiguities.

The β-glycosidase expressed in *Escherichia coli* from one of the clones described above was functional and showed high thermal stability (100% active after 10 min at 85 °C), Topt >90 °C, and a substrate specificity similar to that of the enzyme described previously (*14*). For these reasons this clone, named pWUR8, was considered as the wild type and was used to express the Taβ-gly enzyme described in this paper; furthermore, it was used for the site-directed mutagenesis experiments.

Site-Directed Mutagenesis. The preparation of the Ssβ-glyE387G mutant has been described previously (*11*). The mutants CelBE372A and Taβ-glyE386G were prepared by site-directed mutagenesis from the pLUW511 and pWUR8 plasmids, respectively. Mutation E372A was introduced in CelB by the following oligonucleotides (mismatches are underlined): 5′-ATGATAATTACAGG/CCAACGGTATG-GCC-3′ and 5′- GGCCATACCGTTGG/CCTGTAATTAT-CAT-3′ following a previously described protocol (*18*). The plasmid containing the desired mutation was identified by direct sequencing and completely resequenced; the mutant clone was named pWUR21. Mutation E386G was introduced in Taβ-gly by the following oligonucleotide (mismatch is underlined): 5′-GACTTGATCGTGACCGGGAACGGT-GTTTCAGACAGC-3′ following the method of Higuchi and co-workers (*19*) based on PCR. The restriction fragment SacI-BamHI of 598 bp containing the mutation was obtained from the DNA amplification and substituted for the corresponding fragment in the wild-type pWUR8 plasmid. The plasmid containing the desired mutation was identified by direct sequencing and completely resequenced; the mutant clone was named pWURE386G.

Enzyme Expression and Purification. Wild-type Ssβ-gly and the mutant Ssβ-glyE387G were expressed and purified as described previously (*11*). Wild-type Taβ-gly and mutant Taβ-glyE386G were expressed from *E. coli* BL21(DE3)RIL cells, carrying the plasmids pWUR8 and pWURE386G, respectively, grown in SB medium at 37 °C. Gene expression was induced by the addition of 1 mM IPTG when the culture reached an OD_{600} of 1.0. Growth proceed for 16 h, and cells were harvested by centrifugation at 5000*g* and frozen at −20 °C. The resulting cell pellet was thawed, resuspended in 2 mL g^{-1} cells of 50 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl (PBS buffer) and homogenized by treatment with cell disruption equipment (Constant Systems Ltd., Warwick, UK). After disruption, the homogenate was centrifugated for 30 min at 30 000*g* at 4 °C; cell debris were discarded, and the crude extract was incubated for 30 min at 75 °C and centrifuged again at the same conditions. The pellet containing the denatured proteins was discarded, and the supernatant was incubated again for 10 min at 85 °C and then centrifuged at the same conditions; the protein pellet was discarded. After this treatment, 1 M ammonium sulfate was added to the supernatant, and the sample was directly loaded onto a phenyl-sepharose FPLC column (Amersham Biotech) equilibrated with 50 mM sodium phosphate buffer pH 6.8 and 1 M ammonium sulfate. Three volumes of a linear gradient of this buffer against water were used to elute the column; wild-type and mutant proteins elute in a single fraction of 5 mL. This purification procedure yielded about 3.0−4.0 mg of pure protein from 10 to 15 g of wet cell pellet.

Wild-type CelB and the mutant CelBE372A were expressed and purified from *E. coli* BL21(DE3)RIL cells, carrying the plasmids pLUW511 and pWUR21, respectively, as described for Taβ-gly. In this case, the heat treatment was performed by incubating the crude extract for 30 min at 80 °C and centrifugated for 30 min at 30 000*g* at 4 °C. Thereafter, the hydrophobic chromatography was performed as described above. This purification procedure yielded about 20−25 mg of pure protein from 20 to 25 g of wet cell pellet.

All the enzymes purified were >95% pure by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and were used for all the subsequent characterization. To eliminate any trace of wild-type contaminating activity in the preparations of the β-glycosidase mutants, samples were incubated with the 2,4-dinitrophenyl-2-deoxy-2-fluoro-β-glucoside (2,4-DNp-2F-β-Glc), a mechanism-based inhibitor, as previously reported (*11*). The protein concentration was determined with the method of Bradford (*20*), by using bovine serum albumin as standard. The samples stored at 4 °C in sodium phosphate 20 mM pH 6.5 buffer are stable for several months.

Enzyme Characterization. All kinetic studies were performed by following changes in UV/vis absorbance with a Varian Cary 100 Scan spectrophotometer (Australia) thermally controlled by a Peltier system. Quartz cuvettes were preheated for 2 min, and temperature was kept constant during all activity measurements. The standard assays for the β-glycosidase activities were performed at 65 °C in 50 mM sodium phosphate buffer at pH 6.5, in the presence of 2-Np-Glc substrate at 5 mM final concentration. Kinetic parameters at standard conditions were measured by using 2-Np-Glc substrate concentrations ranging from 0.02 to 20 mM. Wild-type and mutant β-glycosidase amounts ranging from 0.5 to 20 μg were used in each assay. A blank mixture, containing all the reactants except the enzyme, was used in the characterization to correct the spontaneous hydrolysis of the substrates. The molar extinction coefficient (ϵ_M) value of 1711 M^{-1} cm^{-1} at 405 nm for 2-nitrophenol was measured at 65 °C in 50 mM sodium phosphate buffer pH 6.5 and used for the calculation of the enzymatic activity. One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of substrate in 1 min at 65 °C, under standard conditions.

Reactivation studies in the standard assay conditions were performed by adding sodium formate at the concentration indicated. The activity of mutant β-glycosidases at acidic conditions was measured in 50 mM sodium formate/formic acid buffers in the pH range of 3.0−6.0. Kinetic parameters were measured by using 2-Np-Glc substrate concentrations ranging from 0.1 to 30 mM. The molar extinction coefficients of 2-nitrophenol, measured at 405 nm and 65 °C in 50 mM sodium formate buffer in the pH range of 3.0−6.0, were the following: pH 3.0, 403 M^{-1} cm^{-1}; pH 4.0, 423 M^{-1} cm^{-1}; pH 5.0, 480 M^{-1} cm^{-1}; and pH 6.0, 713 M^{-1} cm^{-1}. The ϵ_M values of 4-nitrophenol and 2,4-dinitrophenol at 405 nm, measured at 65 °C in 50 mM sodium formate buffer pH 3.0, were 73.6 and 2196 M^{-1} cm^{-1}, respectively.

The activity of mutant β-glycosidases on cellobiose was performed by incubating the enzymes in 50 mM sodium formate buffer pH 3.0, at 65 °C, in the presence of 200 mM substrate; after 10 min incubation, the glucose released was measured with the glucose oxidase-peroxidase enzymatic assay (GOD-POD). All the kinetic data were plotted and refined using the program Grafit (*21*).

The stability of the mutant β-glycosidases was analyzed by incubating the enzymes at the concentration of 20 μg mL^{-1} in 50 mM sodium formate buffer pH 3.0 or 4.0 at 65 °C. Samples were taken at proper times, and residual β-glycosidase activity was measured on 2-Np-Glc in 50 mM sodium formate buffer pH 4.0, at 65 °C; the activity of the sample measured in the same conditions before incubation was taken as 100%.

Enzymatic Synthesis of Oligosaccharides. A total of 5 mg (0.0166 mmol) of 2-Np-Glc dissolved in 50 mM sodium formate pH 4.0 was reacted with 21.5 μg of the hyperthermophilic glycosynthases at 65 °C (1.29 μg of protein/μmol of substrate). The 50 μL samples were withdrawn at 5 min intervals within 3 h reaction time and enzymatically analyzed (GOD-POD) for free glucose content. An appropriate blank experiment was conducted in parallel to correct the values for spontaneous hydrolysis of 2-Np-Glc. TLC were developed in EtOAc/MeOH/H_2O, 70:20:10 by volume. HPLC and NMR studies for the analysis of the disaccharidic portion were conducted as reported (*12*) after preparative TLC and acetylation of disaccharides. Reactions at pH 3.0 and 5.0 in sodium formate 50 mM buffer were performed in similar conditions. Selective NMR signals of acetylated derivatives of disaccharides of 2-Np are as follows: β-1-3 (^{1}H and ^{13}C NMR signals, benzene-d_6) δ 4.49 (101.4), 5.42 (72.5), 3.84 (78.7), 4.92 (68.6), 3.34 (72.1, 4.09−4.14 (62.04) (internal glucose)) and 4.56 (100.8), 5.12 (72.2, 5.30 (73.34), 5.13 (68.4), 3.25 (71.6), 4.35−3.94 (61.8)) (external glucose). β-1-6 (^{13}C NMR signals, $CDCl_3$) δ 100.2, 72.7, 72.04, 68.7, 74.0, 68.1 (internal glucose) and 100.6, 72.3, 71.1, 68.2, 70.4, 61.7 (external glucose). β-1-4 (anomeric ^{1}H and ^{13}C NMR signals, $CDCl_3$) δ 4.55 (100.8, 72.2, 72.07, 75.92, 70.48, 61.45 (internal glucose)) 5.14 (99.2, 72.8, 72.9, 67.7, 70.9, 61.45).

RESULTS

Cloning and Expression of the T. aggregans β-Glycosidase. The gene encoding for Taβ-gly was cloned from the *T. aggregans* genomic DNA. All the isolated clones obtained from two independent experiments revealed the mutations N62Y, Q63K, L231F, V303A, and A412V, if compared with the sequence Q9YGA8 deposited in the data bank. Furthermore, direct sequencing of the products obtained by amplification of the *T. aggregans* genome confirmed these mutations with no ambiguities; nevertheless, the gene carrying these mutations expressed a functional β-glycosidase with characteristics similar to the enzyme described by Chi and co-workers (*14*). The location of the residues N62, Q63, L231, V303, and A412 on the surface of the native tetramer of Taβ-gly (Figure 1) may explain why these mutations did not influence the properties of the enzyme. The presence of the same mutations in six independent clones obtained from two different experiments and in the PCR product suggests that they naturally occurred in our *T. aggregans* strain and were not introduced during the amplification of the genomic DNA. For these reasons, we considered this gene as the wild

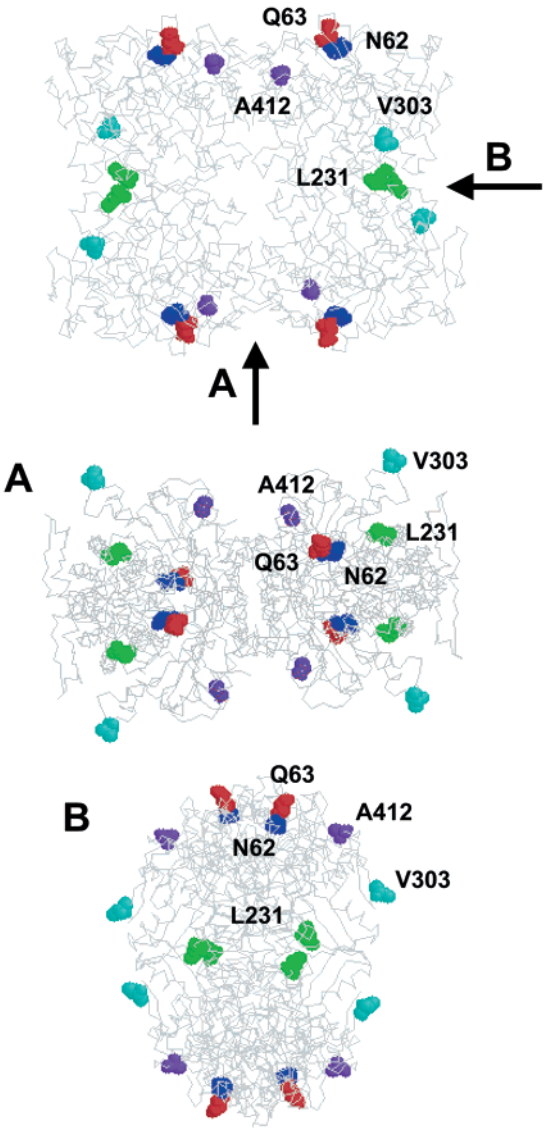


FIGURE 1: 3-D structure of the β-glycosidase from *Thermosphaera aggregans* (PDB 1QVB). The space fill of the residues N62 (blue), Q63 (red), L231 (green), V303 (cyan), and A412 (purple), which resulted from being changed to Y62, K63, F231, A303, and V412, respectively, in the enzyme utilized in the present paper, are reported in the front, the bottom (A), and the right (B) side views, respectively. This figure was generated with the program RasMol 2.6.

Table 1: Steady-State Kinetic Constants of the Wild-Type β-Glycosidases and of the Reactivated Mutants[a]

	reaction conditions	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	% reactivation[b]
Taβgly		0.20 ± 0.06	309.0 ± 18.5	1575.7	
	+ formate 2 M	0.38 ± 0.07	439.8 ± 16.4	1164.6	
TaβglyE386G		ND[c]	ND	ND	
	+ formate 2 M	1.01 ± 0.10	72.9 ± 2.4	72.9	4.60
Ssβgly		1.01 ± 0.24	538.0 ± 11.0	533.0	
	+ formate 2 M	0.50 ± 0.07	425.0 ± 11.0	850.0	
SsβglyE387G		ND	ND	ND	
	+ formate 2 M	1.17 ± 0.12	53.0 ± 1.2	45.0	8.44
CelB		0.28 ± 0.06	1796.9 ± 90.2	6480.0	
	+ formate 2 M	0.22 ± 0.08	1189.0 ± 94.1	5350.9	
CelBE372A		ND	ND	ND	
	+ formate 2 M	1.49 ± 0.12	3.9 ± 0.1	2.6	0.04

[a] Kinetic constants were measured in 50 mM sodium phosphate buffer pH 6.5. [b] % reactivation is calculated by taking as 100% the k_{cat}/K_M of the wild-type β-glycosidases assayed at standard conditions. [c] ND (not detectable) means that, using concentrations of enzyme of 10 μg/mL in the assay, the rates of change in absorbance did not vary in the experimental conditions and were approxymately the same as in the control without enzyme.

type, and we used it to express the Taβ-gly enzyme described in the following characterization and to prepare the Taβ-glyE386G mutant. The expressed wild-type Taβ-gly exhibited a specific activity on 2-Np-Glc of 129.6 U mg^{-1} at 65 °C in standard conditions.

Preparation and Characterization of Mutated β-Glycosidases. The nucleophile residues E386 and E372 in the Taβ-gly and CelB enzymes, respectively, were changed, by site-directed mutagenesis, to a non-nucleophile residue. The obtained Taβ-glyE386G and CelBE372A mutants and the corresponding wild-type enzymes were purified by two simple purification steps consisting in a fractionaction at high temperature and hydrophobic chromatography. Along the purification procedure, the mutant enzymes showed a behavior similar to that of their corresponding wild types, suggesting that the mutations introduced did not affect significantly the stability of these proteins at the conditions used.

Assays on 2-Np-Glc substrate at 65 °C of the Taβ-glyE386G and CelBE372A mutants showed that the mutations completely abolished the activity of the two enzymes; this was not surprising considering the non-nucleophilic nature of the residues introduced. However, the addition of 2 M sodium formate as an external nucleophile led to the recovery of 4.60% activity with the Taβ-glyE386G mutant and about 0.04% of wild-type activity with the CelBE372A mutant (Table 1). The reactivation observed with the Taβ-glyE386G mutant is comparable to that previously reported with the hyperthermophilic glycosynthase Ssβ-glyE387G, which is also shown in Table 1 (*22*).

Preliminary analyses by thin-layer chromatography (TLC) of the reaction mixture containing the mutated β-glycosidases Taβ-glyE386G and CelBE372A in the presence of 2-Np-Glc and 4 M sodium formate at 65 °C showed the conversion of the substrate and the accumulation of oligosaccharides (not shown) as observed by using the Ssβ-glyE387G mutant (*12*). These data indicated that Taβ-glyE386G and CelBE372A behave as hyperthermophilic glycosynthases; the accumulation of the products was notably faster with Taβ-glyE386G and Ssβ-glyE387G than with CelBE372A.

Glycosynthases Characterization at Acidic pHs. The activity of the Ssβ-glyE387G, Taβ-glyE386G, and CelBE372A mutants at acidic pHs was assayed on 5 mM 2-Np-Glc in 50 mM sodium formate buffer in the pH range of 3.0−6.0 at 65 °C. The highest rates were observed at the lowest pH tested; also, in these conditions the glycosynthase from *P. furiosus* was about 20-fold less active than the *S. solfataricus* and *T. aggregans* mutants (not shown). The high activity of these mutants at a pH below 5.0 contrasts the broad pH optimum (between 5.5 and 6.5) that we have found for the wild-type Ssβ-gly and CelB enzymes (*16*). No activity could be obtained for all the mutants in the presence of sodium citrate and sodium acetate buffers 50 mM, pH 3.0, indicating that the nature of the external nucleophile plays a critical role in the reactivation of these enzymes.

Remarkably, the three mutants were fully stable for 2 h at 65 °C in 50 mM sodium formate buffer, pH 4.0 (Figure 2). At pH 3.0, Ssβ-glyE387G and CelBE372A mutants after 90 min showed more than 50 and 80% residual activity, respectively, whereas after 10 min the activity of the Taβ-glyE386G mutant is only 5% of that observed before incubation.

From these results we decided to measure the kinetic constants, at the conditions described, in the first minute of the assay in which all the mutants maintained at least 90% activity (Table 2). At pH 5.0, all the mutants showed turnover numbers similar to those found at pH 6.5 in 2 M sodium formate (Table 1), but the values increased sharply up to the maximal value observed at pH 3.0. Even for the less active CelBE372A mutant, we obtained at pH 3.0 a k_{cat} similar to those of the Ssβ-glyE387G and Taβ-glyE386G mutants at pH 5.0.

Substrate Specificity of Hyperthermophilic Glycosynthases. Ssβ-glyE387G, Taβ-glyE386G, and CelBE372A mutants were assayed at 65 °C in 50 mM sodium formate buffer pH 3.0 on a variety of substrates: aryl-glucosides containing groups that differ in their pK_a and therefore in their leaving ability, two 2-Np-glycosides, and cellobiose (Table 3). The three mutants showed the highest specific activity on 2,4-DNp-Glc, which has the best leaving group (2,4-dinitrophenol pK_a = 3.96 (*23*)), and on 2-Np-Glc, which shows a leaving group with a pK_a similar to 4-nitrophenol (7.22 and 7.18, respectively (*23*)) but that can form a chelate ring by H-bonding increasing the leaving ability upon protonation (*11*). Remarkably, all the mutants recovered significant activity on 2-Np-Gal and -Xyl; in addition, Ssβ-glyE387G

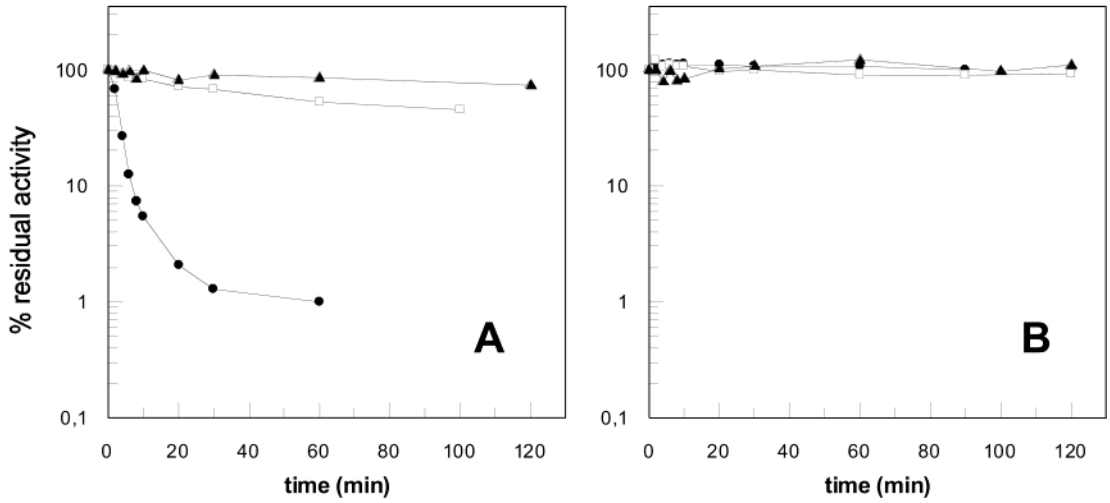


FIGURE 2: Stability of hyperthermophilic glycosynthases. The residual activity at 65 °C in 50 mM sodium formate buffer pH 3.0 (A) and pH 4.0 (B) of Ssβ-glyE387G (open squares), Taβ-glyE386G (closed circles), and CelBE372A (closed triangles) is reported.

Table 2: Steady-State Kinetic Constants of the Hyperthermophilic Glycosynthases in 50 mM Sodium Formate Buffer

	Taβgly E386G			*Ss*βgly E387G			CelB E372A		
reaction conditions	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
pH 3.0	6.6 ± 0.6	970.0 ± 39.9	147.4	16.4 ± 1.6	901.4 ± 32.9	55.0	4.3 ± 0.2	47.6 ± 0.9	10.9
pH 4.0	3.3 ± 0.5	305.3 ± 13.6	91.0	4.9 ± 0.5	321.9 ± 12.0	65.9	2.1 ± 0.4	6.7 ± 0.4	3.1
pH 5.0	3.1 ± 0.4	53.9 ± 2.0	17.2	2.2 ± 0.5	52.9 ± 3.1	23.8	1.3 ± 0.1	0.45 ± 0.01	0.3
pH 6.0	2.8 ± 0.5	11.6 ± 0.6	4.2	1.1 ± 0.4	20.7 ± 1.6	18.5	ND[a]	ND	ND

[a] ND (not detectable); see Table 1.

Table 3: Substrate Specificity of Wild-Type β-Glycosidases and Reactivated Mutants

	Taβgly (U mg^{-1})		*Ss*βgly (U mg^{-1})		CelB (U mg^{-1})	
substrates[a]	wt	E386G	wt	E387G	wt	E372A
2,4-DNp-Glc	75.0 ± 5.0	207.1 ± 9.6	195.6 ± 29.1	126.9 ± 7.2	320.2 ± 77.2	8.3 ± 0.4
2-Np-Glc	72.2 ± 0.1	129.8 ± 2.8	252.6 ± 10.7	63.3 ± 0.2	534.3 ± 31.1	4.7 ± 0.2
4-Np-Glc	61.7 ± 13.3	12.0 ± 0.1	149.8 ± 0.8	14.9 ± 0.1	290.8 ± 27.1	ND[b]
2-Np−Gal	140.9 ± 9.0	6.3 ± 0.6	218.6 ± 6.3	2.7 ± 0.3	1048.6 ± 195.8	0.08 ± 0.01
2-Np-Xyl	10.4 ± 0.7	6.4 ± 0.6	19.1 ± 0.2	2.5 ± 0.1	75.2 ± 4.7	0.28 ± 0.03
cellobiose	33.8 ± 2.6	0.39 ± 0.02	61.9 ± 0.5	1.2 ± 0.1	65.6 ± 4.5	ND

[a] Aryl-glycoside and cellobiose substrates were assayed at 5 and 200 mM final concentration, respectively, in 50 mM sodium formate buffer pH 3.0. [b] ND (not detectable); see Table 1.

and Taβ-glyE386G mutants could hydrolyze also the disaccharide cellobiose.

Synthesis of Oligosaccharides. The efficiency of the formation of 2-Np-oligosaccharide reactions promoted by Ssβ-glyE387G, Taβ-glyE386G, and CelBE372A mutants from the substrate 2-Np-Glc acting both as donor and acceptor, in the presence of 50 mM sodium formate buffer pH 4.0, at 65 °C was investigated by TLC and by GOD-POD up to complete consumption of the substrate. The amount of free monosaccharide found indicates the efficiency of the transfer reaction to an acceptor. TLC quickly allows one to monitor the formation of oligosaccharides and the degree of polymerization in the reaction. Blank experiments show 11% chemical hydrolysis of 2-Np-Glc after 24 h; this value is less than 1% within a 3 h reaction time (not shown).

The most efficient reaction is catalyzed by Ssβ-glyE387G, which, after 30 min, converted almost all (91%) 2-Np-Glc into products (Figure 3). In fact, glucose monitoring indicated that ca. 8% of the free monosaccharide is present in the reaction mixture after this time (Figure 4). With Taβ-glyE386G, after 1 h of reaction, the 2-Np-Glc was completely consumed (Figure 3), with the most part converted into products (70%) since in this case about 30% of the free glucose was found in the reaction mixture (Figure 4). The reaction rate using CelBE372A was much lower; after 7 h the substrate is still present in the reaction mixture (Figure 3).

After 24 h, 60 and 100% of glucose is detected as free monosaccharide in Ssβ-glyE387G and Taβ-glyE386G reaction mixtures, respectively, and 86% of the free glucose is detected in mixtures containing CelBE372A (not shown). Hence, the remaining glucose is present as tetrasaccharides and higher or as a mixture of substrate and disaccharidic product for Ssβ-glyE387G and CelBE372A, respectively.

The TLC analysis of the reaction mixtures at various time intervals (Figure 3) revealed a high degree of polymerization (tri- and tetrasaccharides) for the products of Ssβ-glyE387G, while the most abundant products of Taβ-glyE386G are in the di- and trisaccharidic range. The reactions performed by using Ssβ-glyE387G gave results similar to those obtained

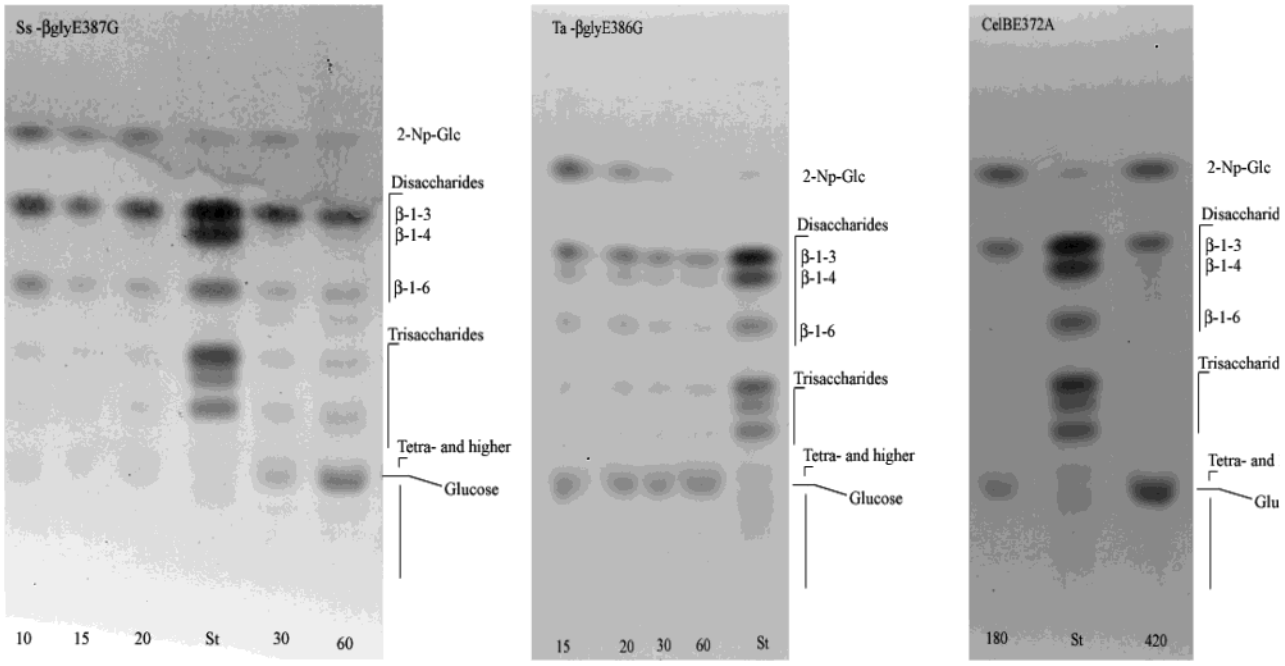


FIGURE 3: Analysis of the synthetic products of the hyperthermophilic glycosynthases. TLC of the reaction mixtures obtained by using Ssβ-glyE387G (left panel), Taβ-glyE386G (central panel), and CelBE372A (right panel). See text for details.

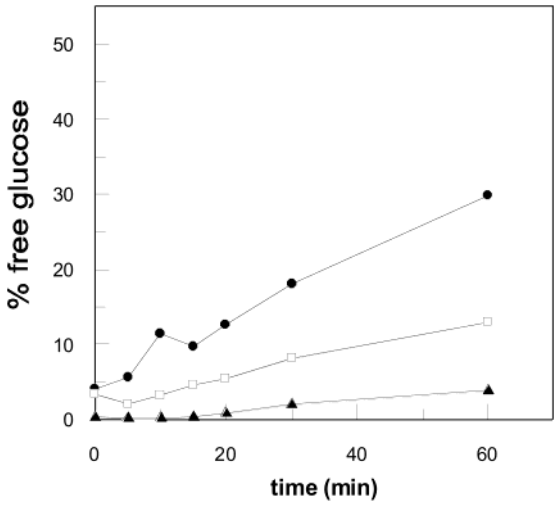


FIGURE 4: Analysis of the free glucose produced by the hyperthermophilic glycosynthases. The glucose produced in the same reaction mixtures of Figure 3, obtained by using Ssβ-glyE387G (open squares), Taβ-glyE386G (closed circles), and CelBE372A (closed triangles), is reported. The total amount of 2-Np-Glc substrate (16.6 mmol) is considered equivalent to 100% glucose.

at 4 M sodium formate (*12*). Taβ-glyE386G formed a mixture of di- (86%), tri- (8%), and tetrasaccharides (5%) as indicated by HPLC. The disaccharides were characterized by HPLC and NMR as β-1-3 (59%), β-1-4 (28%), and β-1-6 (12%) glucose dimers β-O-linked to 2-nitrophenol. Interestingly, the composition of regioisomers was different from that found with Ssβ-glyE387G (80% β-1-3, 2% β-1-4, and 18% β-1-6) (*12*). The trisaccharidic portion is formed by a mixture of products comigrating with the one obtained from the same reaction conducted with Ssβ-glyE387G at 4 M sodium formate (*12*). No effort was made to establish fine structural details of these tri- and tetrasaccharidic compounds. Similar results were obtained at pH 3.0 and 5.0 (not shown). Finally, CelBE372A produced mainly the β-1-3 disaccharide at all pHs tested (3.0, 4.0, and 5.0).

DISCUSSION

The identification of more effective glycosynthases with higher catalytic activity and different specificity is highly desirable to improve the reactivity of these enzymes on donors containing groups with poor leaving ability. In particular, hyperthermophilic glycosynthases able to work at a lower concentration of external nucleophile and at high temperatures would be very attractive to facilitate the product monitoring and to increase the solubility of substrates and products and to improve the purification yields. To this aim, we prepared two new hyperthermophilic glycosynthases, and we explored the synthetic abilities of these novel enzymes at acidic pH.

The codons corresponding to the catalytic nucleophiles Glu372 and Glu386 of CelB and Taβ-gly, respectively, were identified by aligning the amino acid sequences of family 1 glycoside hydrolases (*24*) and were altered by site-directed mutagenesis to encode Ala and Gly, respectively. As expected from the nonnucleophilic nature of the residue introduced, the mutated β-glycosidases had no measurable activity on 2-Np-Glc (Table 1). However, as we previously reported with the Ssβ-glyE387G mutated in the nucleophile of the reaction, the hydrolytic activity of Taβ-glyE386G and CelBE372A was partially restored by using the external nucleophile sodium formate at high concentrations (Table 1). The reactivation rates of *T. aggregans* and *S. solfataricus* mutants were comparable, whereas the CelBE372A mutant recovered only 0.04% of wild-type catalytic efficiency. It is worth noting that, although *P. furiosus* and *S. solfataricus* grow optimally at 100 and 80 °C, respectively, wild-type CelB has a hydrolytic activity higher than that of Ssβ-gly at 60 and 65 °C, respectively (*16*). In this case, the microorganisms' optimal growth temperature does not correlate to the optimal temperature of the β-glycosidases; therefore, the temperature used in our assays does not bias the reactivation in favor of Ssβ-glyE387G. This suggests that the reduced reactivation is the result of the mutant Ala residue in the

active site of CelBE372A, which could limit the access of formate for steric or hydrophobic hindrance. Interestingly, Gly mutations produced more efficient synthases if compared to Ala and Ser mutations in both hyperthermophilic and conventional enzymes (*10*, *22*, *25*), although they follow different mechanisms of reactivation (Scheme 1B,C).

In the presence of an excess of external nucleophile, the mutants Taβ-glyE386G and CelBE372A produced a pattern of oligosaccharidic products similar to that previously observed with Ssβ-glyE387G (*12*), demonstrating that they act as hyperthermophilic glycosynthases.

We analyzed the reactivation rates of the three enzymes in 50 mM sodium formate in the pH range of 3.0−6.0, and remarkably, for all the enzymes we obtained the maximal activity at the lowest pH tested. In particular, the Ssβ-glyE387G and Taβ-glyE386G mutants showed an increment of the k_{cat} of 17- and 18-fold respectively, going from pH 5.0 to 3.0, whereas the CelBE372A mutant showed, between the same pHs, an activation of up to 119-fold (Table 2). In addition, we found that the k_{cat} values of the Ssβ-glyE387G and Taβ-glyE386G glycosynthases at pH 3.0 (Table 2) are 3- and 1.7-fold higher than the values obtained for the Ssβ-gly and Taβ-gly wild types, respectively, assayed at optimal conditions (Table 1). The k_{cat} value obtained with the CelBE372A mutant at pH 3.0 is similar to that observed with the mutants from *S. solfataricus* and *T. aggregans* at pH 5.0. The K_M values increase significantly from pH 5.0 to 3.0 for the three enzymes (Table 2), indicating changes in the affinity for the substrate.

To test the substrate specificity of the glycosynthases, we assayed the enzymes at pH 3.0 on a variety of substrates (Table 3). At these conditions, wild-type enzymes showed comparable activity on 2-, 4-, and 2,4-DNp-Glc substrates. We have previously shown that Ssβ-gly had similar specific activities on these substrates also in sodium phosphate buffer 50 mM pH 6.5, at 65 °C (*11*), suggesting that for the wild-type enzymes the second step of the reaction (Scheme 1A) could be rate limiting for these substrates. In contrast, the ability of the leaving group in the substrate had a direct effect on the observed reactivation rates of the hyperthermophilic glycosynthases, which show the highest rates on 2,4-DNp-Glc (Table 3). This may suggest that the first step of the reaction became limiting for these glycosynthases. However, a more detailed mechanistic study of the wild-type and mutant enzymes on a greater range of donors with different leaving ability would clear these points. It is worth noting that Ssβ-glyE387G and Taβ-glyE386G glycosynthases were active on glucosides showing groups with a poor leaving ability, such as 4-Np-Glc and cellobiose, and that all the enzymes could use as donors 2-Np-gluco, -galacto, and -xylosides. The body of the results shown in Table 3 indicates that the general activation observed at acidic conditions broadened the substrate specificity of the enzymes restoring the characteristics of the wild-type β-glycosidases (*14*, *15*, *26*).

The harsh conditions at which the reactions were performed affected the stability of the enzymes at different extents. At pH 3.0, the Taβ-glyE386G mutant was rapidly inactivated, whereas Ssβ-glyE387G and CelBE372A mutants maintained a significant stability for up to 2 h (Figure 2). However, at pH 4.0 all the mutants remained 100% active as expected from the extremophilic nature of these enzymes, and the Ssβ-glyE387G mutant showed its highest k_{cat}/K_M among the pHs tested.

These observations drove us to adopt 50 mM sodium formate buffer pH 4.0 in synthetic reactions to test if the hyperthermophilic glycosynthases could be exploited for the efficient production of oligosaccharides. In these reactions, the aryl-glucoside acts as both donor and acceptor forming a variety of disaccharides. As previously reported, these products become acceptors during the reaction producing various linear and branched oligosaccharides (*12*). By inspection of the reaction mixtures by TLC at different time intervals, and by comparing the amounts of 2-Np-Glc and of free glucose in the reaction mixtures (Figures 3 and 4), we found that the Ssβ-glyE387G mutant showed the highest efficiency in oligosaccharide synthesis. The reaction was complete in 30 min using 1.29 μg of protein/μmol of substrate. The same reaction, conducted at 4 M sodium formate in phosphate buffer pH 6.5, requires twice the amount of time (ca. 60 min) and of glycosynthase enzyme (2.64 μg/μmol of substrate) to obtain similar product yields (*12*). Furthermore, the reactivation in diluted sodium formate solutions greatly facilitates the monitoring and chromatographic workup for the preparative scale-up of syntheses.

The glycosynthases used in these experiments produced different products, with marked differences between Ssβ-glyE387G and Taβ-glyE386G glycosynthases. In particular, Taβ-glyE386G produced mostly di- and trisaccharides, with a 14-fold higher amount of the β-1-4 regioisomer if compared to Ssβ-glyE387G. Instead, Ssβ-glyE387G showed a striking preference to the formation of the β-1-3 bond, as reported previously (*12*). By contrast, the CelBE372A mutant produced only the 2-Np-laminaribioside that, presumably for the low activity of this glycosynthase, could not be present in a sufficient amount to compete as an acceptor with 2-Np-Glc. However, the different product pattern observed could reflect the diversity in the active sites of the three enzymes.

It is worth noting that, after a 24 h incubation of these reaction mixtures, almost all the glucose expected from the 2-Np-Glc was found free in solution, suggesting that the donor, and the products of the reaction identified in the first 60 min, were completely hydrolyzed. Acidic hydrolysis because of the extended incubation time was not responsible of this degradation since the blank mixture still contains ca. 90% of the substrate. The low activity on the disaccharide cellobiose of the Ssβ-glyE387G and Taβ-glyE386G mutants (Table 3), after long incubation, could have been responsible of the hydrolysis of the products. This is a crucial point since the reactivation obtained at acidic conditions could have been so efficient to transform again the glycosynthases in glycoside hydrolases making the exploitation of these enzymes for the synthesis of oligosaccharides questionable. However, the efficiency of the catalyzed reactions shown in Figures 3 and 4 can secure against this question showing that, during the first hour of incubation, the effect of the hydrolytic activity on the products is negligible. Therefore, the hydrolysis of the products occurs only after incubations longer than 3 h.

The reasons of the complete hydrolysis of the products are still obscure; Ssβ-glyE387G and Taβ-glyE386G glycosynthases show large differences in specific activity on 2-Np-Glc and cellobiose substrates with an activation of 53- and 324-fold, respectively, on 2-Np-Glc, with respect to cello-

biose (Table 3). This suggests that they had scarce hydrolytic activity on the oligosaccharides produced in the reaction. As a comparison, the specific activity of Ssβ-gly and Taβ-gly wild types on 2-Np-Glc is only 4- and 2-fold, respectively, higher than that found on cellobiose (Table 3). In the three glycosynthase preparations used in these experiments, we depleted any wild-type contaminating enzymatic activity by incubation with the mechanism-based inhibitor 2,4-DNp-2F-β-Glc. However, we cannot exclude that long incubations at the harsh conditions could have promoted the release of the inhibitor from the active site of the contaminating wild types, which could have hydrolyzed the products.

The studies reported here indicate that the activity of glycosynthases rescued by adding sodium formate is greatly improved at acidic conditions in which the acid/base catalyst is protonated and can perform better the first step of the reaction (Scheme 1C). This indication is supported by the observation that at pH 3.0, at which the three glycosynthases work optimally (Table 2), formate (pK_a 3.75) is present at a concentration lower than that found at pH 4.0 or higher. Therefore, the activation observed is not correlated to the concentration of the nucleophile but rather to the acidity of the reaction mixture. Moreover, the high efficiency observed in the glycosynthetic reaction indicates that the conditions used did not affect the second step of the reaction, in which the acid/base carboxylate should be ionized for best performance (Scheme 1C). Presumably, acidic conditions could have been beneficial also in weakening the stability of the glycosyl-formate ester intermediate.

Our data are in accordance with the recent observation that the activity of a mesophilic glycosynthase was improved by modifying the ionization state of the acid/base catalyst. In fact, the endo-glycosynthase from barley showed, on α-glycosyl-fluoride substrates, a basic shift of its pH optimum 2−3 units higher than that of the wild-type enzyme (*10*). This case follows the same principle described here for hyperthermophilic glycosynthases; for the endo-glycosynthase, basic conditions maintain the acid/base catalyst in the ionized form. This promotes best the glycon transfer, which is the only step of the reaction mechanism proposed for mesophilic glycosynthases (Scheme 1B).

It has been recently pointed out that the glycoside hydrolase activity can be tailored by modifying the pK_a of the catalytic residues (*27*). We described in this work a new method, based on the use of diluted acid buffered sodium formate, that rescues the glycosynthase specific activity at levels comparable to the wild type, improving the efficiency of the synthetic reaction in terms of time and amounts of enzyme used. The success of our approach for three different enzymes indicates that it is of general applicability for glycosynthases from hyperthermophiles that, for their intrinsic stability, can resist to critical reaction conditions such as high temperature, low pH, and high concentrations of organics. This approach could be hampered by the stability of donors and acceptors at the harsh conditions used; however, we observed the formation of oligosaccharides by using hyperthermophilic glycosynthases with 2-Np-Gal (as acceptor and donor) and with 2-Np-Glc as donor and 4-methylumbelliferyl β-D-glucoside or α-D-xyloside of 4-penten-1-ol as acceptors (manuscript in preparation).

Hyperthermophilic enzymes are often considered a useful alternative to conventional enzymes for their biodiversity and their intrinsic stability, which allowed their exploitation in particular applications, as is the case of thermophilic DNA polymerases in PCR. However, the use of the enzymes from a thermophilic source is still limited because biotransformations in harsh conditions are restricted to special cases of demonstrated utility. The approach presented here opens up new strategies for the exploitation of hyperthermophilic glycosynthases in oligosaccharide synthesis.

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