Restoration of the Activity of Active-Site Mutants of the Hyperthermophilic β-Glycosidase from *Sulfolobus solfataricus*: Dependence of the Mechanism on the Action of External Nucleophiles†

Marco Moracci,*,‡ Antonio Trincone,§ Giuseppe Perugino,‡ Maria Ciaramella,‡ and Mosé Rossi‡,||

Institute of Protein Biochemistry and Enzymology,Consiglio Nazionale delle Ricerche, Via Marconi 10, 80125 Naples, Italy, Istituto per la Chimica di Molecole di Interesse Biologico, Consiglio Nazionale delle Ricerche, Via Toiano 2, Arco Felice 80072, Naples, Italy, and Dipartimento di Chimica Organica e Biologica, Università di Napoli "Federico II", Via Mezzocannone 16, 80134 Naples, Italy

Received August 3, 1998; Revised Manuscript Received September 28, 1998

ABSTRACT: The β-glycosidase from the hyperthermophilic Archaeon *Sulfolobus solfataricus* hydrolyzes β-glycosides following a *retaining* mechanism based upon the action of two amino acids: Glu387, which acts as the nucleophile of the reaction, and Glu206, which acts as the general acid/base catalyst. The activities of inactive mutants of the catalytic nucleophile Glu387Ala/Gly were restored by externally added nucleophiles. Sodium azide and sodium formate were used as external nucleophiles and the products of their reaction were characterized. Glu387Ala/Gly mutants were reactivated with 2,4-DNP-β-Glc substrate and the Glu387Gly mutant showed recovered activity, with the same nucleophiles, also on 2-NP-β-Glc. The reaction catalyzed by the Glu387Gly mutant proceeded differently depending on the type of externally added nucleophile. Sodium azide restored the catalytic activity of the mutant by attacking the α-side of the anomeric carbon of the substrates, thereby yielding an *inverting* glycosidase. Sodium formate promoted the opposite behavior (*retaining*) in the mutant, producing 3-*O*-β-linked disaccharide derivative of the substrates. A possible role of sodium formate as a biomimicking agent in replacing the natural nucleophile Glu387 is also discussed.

Glycosyl hydrolases are enzymes for which an increasing amount of structural and biochemical data is becoming available (*1*, *2*). This has recently made possible a new classification of these enzymes in clans or superfamilies on the basis of reaction mechanisms, conserved 3D structures, and amino acid sequence similarities (*3*). Glycosyl hydrolases follow two major mechanisms, one giving the overall retention and the other giving the inversion of the anomeric configuration of the substrate. Both mechanisms involve two functional groups in the active site—one carboxyl, one carboxylate—highly conserved in each family. In *inverting* enzymes (Scheme 1A) these residues, which show an average distance of 9.3 Å, function as an acid and a basic catalyst, respectively, and operate with a single displacement of the leaving group (*1*). Instead, *retaining* enzymes (Scheme 1B), in which the two residues are separated, on average, by 5.0 Å, follow a two-step mechanism with formation of a covalent glycosyl-enzyme intermediate. The carboxyl group in the active center functions as a general acid/base catalyst and the carboxylate as the nucleophile of the reaction (*4*, *5*).

It has been demonstrated that the reaction mechanism of glycosyl hydrolases can be changed by mutagenesis. In the case of T4 lysozyme, the mutation Thr26His, which replaces a residue not directly involved in catalysis with another able to act as a nucleophile, converted the mechanism from *inverting* to *retaining* (*6*). Instead, the β-glucosidase from *Agrobacterium* and the β-1,4-glycanase from *Cellulomonas fimi* were converted from *retaining* into *inverting* enzymes by mutating the nucleophile of the reaction. In both cases, enzyme activity was restored by providing high concentrations of external nucleophiles and only by using 2,4-DNP-based substrates that possess good leaving groups (*7*, *8*).

The β-glycosidase from the Archaeon *Sulfolobus solfataricus* (Ssβ-gly;[1] E.C. 3.2.1) is a hyperthermophilic enzyme with optimal activity at temperatures over 85 °C and remarkable thermostability: half-life at 75 °C > 24 h (*9*). The amino acid sequence places the enzyme in glycosyl hydrolase family 1, which has been classified in superfamily 4/7 and in clan GH-A (*3*, *10*). Ssβ-Gly shows broad

† This work was supported by the EC project Biotechnology of extremophiles, Contract BIO2-CT93-0274, and by the EC FAIR project Enzymatic Lactose Valorization, Contract 1048/96.

* To whom correspondence should be addressed. Tel. +39-81-7257246; fax +39-81-2396525; e-mail: moracci@dafne.ibpe.na.cnr.it.

‡ Institute of Protein Biochemistry and Enzymology, CNR.

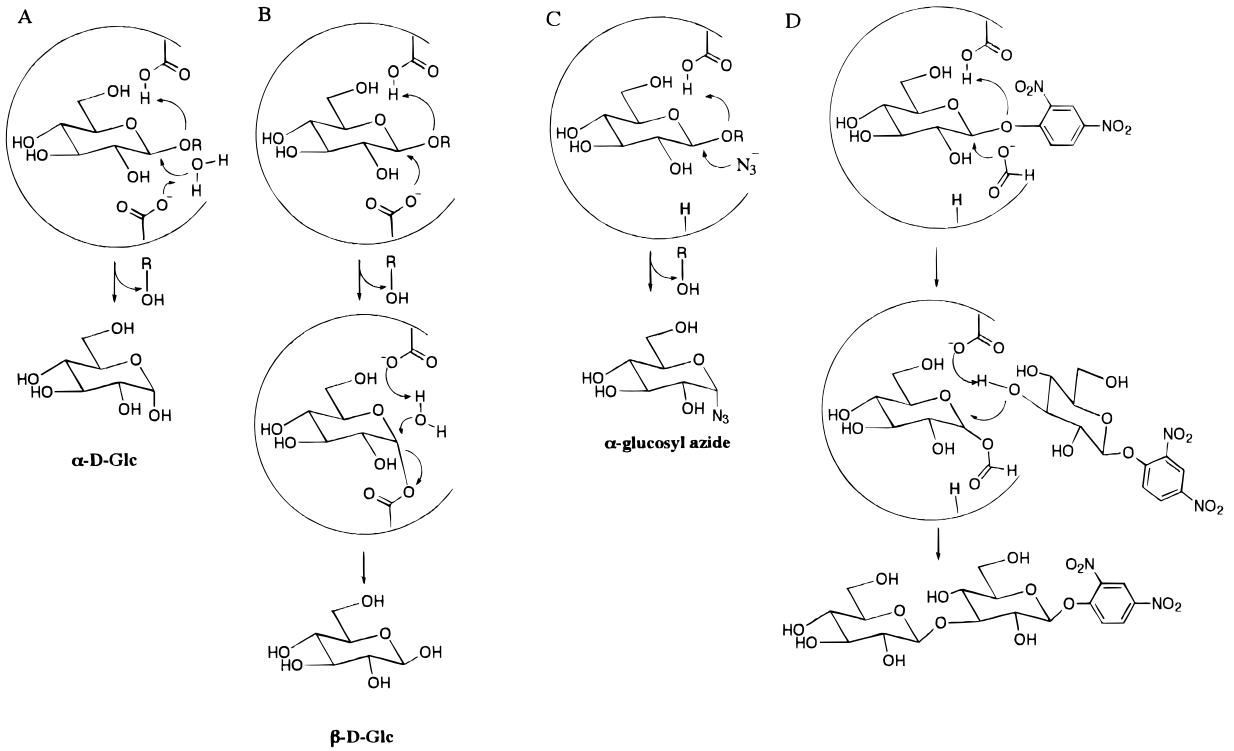
§ Istituto per la Chimica di Molecole di Interesse Biologico, CNR.

|| Università di Napoli "Federico II".

[1] Abbreviations: Ssβ-gly, β-glycosidase from *Sulfolobus solfataricus*; HPLC, high-performance liquid chromatography; RI, refractive index; NMR, nuclear magnetic resonance; GST, glutathione S-transferase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; 2,4-DNP-β-Glc, 2,4-dinitrophenyl-β-D-glucopyranoside; 2-NP-β-Glc, 2-nitrophenyl-β-D-glucopyranoside; 4-NP-β-Glc, 4-nitrophenyl-β-D-glucopyranoside; 2,4-DNP-2F-β-Glc, 2,4-dinitrophenyl-2-deoxy-2-fluoro-β-glucopyranoside; OD, optical density; TLC, thin-layer chromatography; COSY, correlation spectroscopy; FAB-MS, fast atom bombardment mass spectrometry.

10.1021/bi981855f CCC: $15.00
Published on Web 11/10/1998

Scheme 1



substrate specificity for the hydrolysis reaction of several glycosides and β1−3, β1−4, and β1−6 glucose dimers. A remarkable exoglucosidase activity was found against oligosaccharides with up to 5 glucose residues, which are hydrolyzed from the nonreducing end (*11*). This enzyme is also capable of efficiently synthesizing different β-D-glycosides by transglycosylation (*12*). Ssβ-Gly hydrolyzes β-glycosides following a *retaining* reaction mechanism based upon the action of at least two amino acids; namely, Glu387 acting as nucleophile and Glu206 acting as general acid/base catalyst (*13*). The role of Glu387 residue was demonstrated by site-directed mutagenesis and using an active-site-directed inhibitor. Upon substitution of this residue with isosteric Gln, the mutant showed no measurable activity on all the substrates tested; this is the expected result if Glu387 residue is the nucleophile of the reaction, since Gln is unable to promote the nucleophilic attack on the anomeric carbon and to form the covalent α-glycopyranosyl-enzyme intermediate (*13*). Moreover, the incubation with conduritol B epoxide determined complete inactivation of the wild-type enzyme, and the electrospray tandem mass analysis revealed that the inhibitor was covalently bound to Glu387 (*14*). These results were confirmed by direct observation of the Ssβ-gly 3D structure which has been resolved at 2.6 Å (*15*). The glutamic acid 387 is located in the substrate binding site and its side chain is 4.5 Å from the general acid/base catalyst Glu206. These two residues form the catalytic center of the enzyme and their separation is consistent with the *retaining* reaction mechanism.

In this paper we demonstrate that the activity of Glu387Ala/Gly mutants of Ssβ-gly can be restored by the addition of external nucleophiles and that the reaction mechanism depends on the type of nucleophile used. In particular, the Glu387Gly mutant in the presence of sodium azide leads to α-glucosyl azide as the sole product identified by NMR spectroscopy, indicating that it functions as an *inverting* enzyme. Conversely, when sodium formate is added as nucleophile, the same mutant follows a *retaining* mechanism since it produces 3-*O*-β-linked disaccharide derivatives of the substrates. These findings indicate that formate has a biomimetic role restoring the function of the natural nucleophile Glu 387.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of analytical grade. HPLC was performed on a Milton Roy apparatus (England) equipped with UV and RI detectors. NMR spectra were recorded on a Bruker AMX 500 (500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C spectra). Samples for NMR analysis were prepared by dissolving the compounds in the solvents indicated; the corresponding signals were used as internal standards.

Protein Expression and Purification. Wild-type and mutant Ssβ-gly proteins were expressed and purified as fusions of glutathione S-transferase (GST) from vector pGEX-K-Gly, as previously reported (*13*). Briefly, fusion proteins extracted from *Escherichia coli* were purified by affinity chromatography on a glutathione−Sepharose 4B column and incubated with thrombin protease in order to separate GST and Ssβ-gly. Samples were then applied to a Superdex 200 gel-filtration column, which allowed the purification to homogeneity (estimated by SDS−PAGE). Ssβ-Gly enzymes were stored at −20 °C in 20 mM sodium phosphate buffer, pH 7.0, and 50% glycerol (v/v). Protein concentrations were determined by the method of Bradford (*16*), with bovine serum albumin as standard, and spectrophotometrically: a 10 mg/mL Ssβ-gly solution in 0.1 M sodium phosphate buffer, pH 6.5, shows an absorbance of 28.78 OD at 280 nm at room temperature.

Mutagenesis. The preparation of the mutant Glu387Gln has been described elsewhere (*13*). The mutants Glu387Ala and Glu387Gly were prepared by site-directed mutagenesis following the method of Mikaelian and Sergeant (*17*) based on the polymerase chain reaction. Mutants were generated by using one degenerated oligonucleotide for both mutations Glu387Ala and Gly. Mutagenic oligonucleotide, designed according to Kuipers et al. (*18*), was the following (mismatches are underlined): 5′-CTCTATATGTACGTTACTGG/CAAATGGTATTGCGGA-3′. Mutant clones were identified by direct sequencing, and restriction fragments containing the mutation were completely resequenced and substituted for the corresponding fragments in the wild-type pGEX-K-Gly.

Enzyme Characterization. All kinetic studies were performed by following changes in UV/vis absorbance with a Varian Cary 1E spectrophotometer equipped with a circulating water bath. Quartz cuvettes were preheated and temperature was kept constant during all activity measurements. The standard assays of Ssβ-gly activity were performed at 65 °C in 50 mM sodium phosphate buffer at pH 6.5, with 2,4-dinitrophenyl-β-D-glucopyranoside (2,4-DNP-β-Glc) and 2- or 4-nitrophenyl-β-D-glucopyranoside (2- or 4-NP-β-Glc) substrates at final concentrations of 1 and 5 mM, respectively. Wild-type and mutants Ssβ-gly amounts ranging from 0.5 to 5 μg were used in each assay. A blank mixture containing all the reactants except the enzyme was used in all the following characterization in order to correct the spontaneous hydrolysis of the substrates. 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. The following molar extinction coefficients were measured at the wavelengths indicated at 65 °C in 50 mM sodium phosphate buffer, pH 6.5, and used for the calculation of the enzymatic activity: 2-nitrophenol, 1711 M^{-1} cm^{-1}, 405 nm; 4-nitrophenol, 9340 M^{-1} cm^{-1}, 405 nm; and 2,4-dinitrophenol, 9630 M^{-1} cm^{-1}, 400 nm.

Kinetic parameters were measured at the indicated conditions with substrate concentrations ranging from 0.01 to 10 mM for 2,4-DNP-β-Glc and from 0.1 to 18 mM for 2- and 4-NP-β-Glc. The protein concentrations in the reaction mixture were 0.5−1 μg/mL and 2−10 μg/mL for wild-type and Ala/Gly mutants, respectively. Reactivation studies were performed by adding sodium azide or sodium formate at the concentration indicated.

Thermal activity of wild-type Ssβ-gly was analyzed at substrate concentrations of 1 mM (in the range 30−60 °C); 5 mM (65 and 70 °C), and 10 mM (75 °C) for both 2,4-DNP-β-Glc and 2-NP-β-Glc. For the Gly mutant, sodium azide and sodium formate were included in the reaction mixture at the final concentration of 2 M, and only for the 2,4-DNP-β-Glc substrate, the concentration was kept constant (1 mM) in the whole range of temperatures tested (30−75 °C). The molar extinction coefficients used were accurately measured at the different temperatures in 50 mM sodium phosphate buffer, pH 6.5. The activation enthalpy ($\Delta H^{\ddagger}$) for each temperature was calculated according to

$$\Delta H^{\ddagger} = E_a - RT$$

where R (8.314 J K^{-1} mol^{-1}) is the universal gas constant and T is the absolute temperature. The activation free energy ($\Delta G^{\ddagger}$) of the reaction was calculated for each temperature according to

$$\Delta G^{\ddagger} = -RT \ln (k_{cat}\, h/kT)$$

where h (6.6256×10^{-34} J s) is Planck's constant and k (1.3805×10^{23} J K^{-1}) is the Boltzmann constant. All kinetic data were plotted and refined with the program *GraFit* (*19*).

Analysis and Inhibition of the Glu387Gly Mutant Preparation for Wild-Type Contamination. The wild-type Ssβ-gly (0.090 mg/mL, 0.358 nmol) was incubated in 50 mM sodium phosphate buffer, pH 6.5, and assayed on 2- and 4-NP-β-Glc as substrates at standard conditions at 65 °C. After addition of 38.8 μM (38.8 nmol, 102:1 inhibitor/enzyme equivalent ratio) of 2,4-dinitrophenyl-2-deoxy-2-fluoro-β-glucoside (2,4-DNP-2F-β-Glc), the mixture was incubated at 50 °C. After incubation, aliquots were assayed again on the same substrates. Complete inactivation was observed after overnight incubation. A mixture containing all the reactants except the inhibitor was also analyzed: wild-type activity was unaffected by 24 h exposure to 50 °C.

Similarly, Glu387Gly mutant (0.141 mg/mL, 0.558 nmol) was incubated in 50 mM sodium phosphate buffer, pH 6.5, in the presence of 57 μM (57 nmol) or 0.33 μM (0.33 nmol) 2,4-DNP-2F-β-Glc (100:1 and 0.6:1 equivalent ratios, respectively). Samples were assayed at standard conditions at 65 °C on 2- and 4-NP-β-Glc as substrates, with and without sodium azide, before and after addition of the inhibitor, as described for the wild type. The mixtures containing all the reactants except the enzyme were monitored at 400 nm and no change in absorbance was observed.

Chemical Synthesis of 2,4-Dinitrophenyl-β-D-glucoside. The synthesis was performed as previously reported (*20*). A solution of glucose (500 mg, 2.8 mmol) and sodium bicarbonate (500 mg, 5.9 mol) in water (5 mL) was immediately treated with a freshly prepared solution of 1-fluoro-2,4-dinitrobenzene (2 equiv) in ethanol (5−10 mL). The reaction mixture was stirred under dark at 20 °C overnight and the product was purified. The glucoside was purified by flash chromatography and crystallized by acetone. ^{1}H NMR, 400 MHz, CD_3OD δ 7.75, 8.50, 8.76 (3 H, aromatic protons), 5.29 (1 H, anomeric proton, $J = 7.34$ Hz), 3.59, 3.45, 3.52−3.53, 3.75, 3.93 (6 H, H2−H6 of sugar). ^{13}C NMR δ 118.8, 122.0, 129.6 (aromatic methine signals); 155.7, 142.7, 141.0 (quaternary aromatic carbons); 102.1, 78.6, 70.6, 74.4, 77.64, 62.2 (glucose carbon signals). The $[\alpha]^{20}_D$ value and the melting point are in accord with those reported. HPLC conditions: water/methanol 8:2 (v/v); UV detector 254 nm; column Lichrocart Superspher 100 RP-18.

The anomerically ^{13}C-labeled compound (7.2 mg) was synthesized as above, starting from [1-^{13}C]glucose (18 mg), but purification was obtained by preparative TLC ($CHCl_3$/CH_3OH/H_2O, 65:25:4 v/v/v).

Enzymatic Synthesis of α- and β-Glucosyl Azides. Sodium azide (2.6 g, 40 mmol) dissolved in 7 mL of 50 mM sodium phosphate buffer, pH 6.5, was added to 6.8 mg (0.02 mmol) of 2,4-DNP-β-Glc. Glu387Gly enzyme (20 μg) was added, and the reaction was started at 75 °C until the glucoside consumption (20 min). A preliminar purification was obtained by silica gel column chromatography ($CHCl_3$/CH_3OH/H_2O, 65:25:4 v/v/v); the fraction so obtained was further purified by preparative TLC. ^{1}H NMR (400 MHz, D_2O) δ

5.52 (1 H, d, $J = 4.1$ Hz), 3.7−3.9 (2 H, m), 3.58−3.69 (2 H, m), 3.55 (1 H, dd), 3.42 (1 H, t). ^{13}C NMR 90.8, 75.1, 74.1, 72.2, 70.1, 61.4 (*21*). Acetylation of this compound gave rise to 2,3,4,6-tetra-*O*-acetyl-α-D-glucosyl azide as indicated by inspection of ^{1}H and ^{13}C NMR spectra, the signals of which were assigned by COSY experiment (5.59 H1, 4.93 H2, 5.36 H3, 5.04 H4, 4.14 H5, 4.25−4.15 H6a/H6b). FAB-MS spectrum *m*/*z* 374 ($M + H^+$).

Sodium azide (2.16 g, 33.2 mmol) and 4-NP-β-Glc (100 mg, 0.33 mmol) were dissolved in 4 mL of 50 mM sodium phosphate buffer, pH 6.5. After addition of wild-type enzyme (1 mL, 2.67 mg/mL), the reaction was started at 75 °C. At donor consumption (2 h) the reaction mixture was rotary-evaporated and the product was purified by silica gel chromatography, producing 8 mg (0.039 mol, 12%) of β-glucosyl azide. ^{1}H NMR (400 MHz, D_2O) δ 4.72 (1 H, d, $J = 8.8$ Hz), 3.7−3.9 (2 H, m), 3.30−3.59 (3 H, m), 3.25 (1 H, t). ^{13}C NMR 91.4, 79.4, 77.3, 74.2, 70.5, 61.9. Acetylation of this compound gave rise to 2,3,4,6-tetra-*O*-acetyl-β-D-glucosyl azide as indicated by inspection of proton and ^{13}C NMR spectra, the signals of which were assigned by COSY and ^{1}H−^{13}C correlation (4.64 H1, 4.95 H2, 5.23 H3, 5.17 H4, 3.80 H5, 4.13−4.26 H6a/H6b; 87.92 C1, 70.66 C2, 72.62 C3, 67.90 C4, 74.04 C5, 61.67 C6).

Identification of Reaction Products of Mutant Enzyme Glu387Gly in the Presence of Sodium Formate. 2,4-DNP-β-Glc (16 mg, 0.046 mmol) was dissolved in 1 mL of 50 mM sodium phosphate buffer, pH 6.5; 4.6 mmol (314 mg) of sodium formate was added and the reaction was started at 75 °C by adding 170 μL (167 μg) of Glu387Gly mutant enzyme. At the end of reaction, monitored by TLC for the substrate consumption, reaction mixture was preliminarily purified by silica gel column chromatography ($CHCl_3/CH_3OH/H_2O$, 65:25:4 v/v/v). The fractions containing the UV−visible products were collected and further purified by preparative TLC. Selected ^{1}H and ^{13}C NMR D_2O/CD_3OD 8:2 (v/v): ^{1}H NMR δ 5.40 (1 H, $J = 7.36$ Hz), 4.78 (1 H, $J = 8.03$ Hz) anomeric protons; 7.48, 8.50, 8.80 (3 H, aromatic protons); 104.7, 101.9 (anomeric carbons); ^{13}C NMR δ 74.2, 75.3 (C2 and C2′); 85.5 (C3); 69.4, 71.52 (C4 and C4′); 78.0, 77.9, 77.4 (C5, C3′, C5′); 62.6, 62.2 (C6 and C6′). Primes indicate the carbons of the external glucose moiety. The assignment was based on the glycosylation shift rule, taking into account α- and β-shifts by a careful comparison with signals of 2,4-DNP-β-Glc. Acetylation of this compound and purification of acetylated product gave rise to a peracetylated derivative whose positive FAB-MS spectrum (*m*/*z* 825, $M + Na^+$; 619, cleavage of glycosidic bond) is in accordance with the structure proposed.

^{13}C NMR Study of Enzymatic Reaction: (A) Enzymatic Hydrolysis by Wild-Type Enzyme. Anomerically ^{13}C-labeled 2,4-DNP-β-Glc (4 mg, 0.01 mmol) was dissolved in 500 μL of 50 mM sodium phosphate buffer, pH 6.5 in D_2O, and a satisfactory ^{13}C NMR spectrum was obtained after 50 scans. Spectra were calibrated by using the appropriate value for the anomeric signal. Wild-type enzyme (20 μL, 2.67 mg/mL) was added and different spectra were obtained at time intervals as shown in Figure 2.

(B) Enzymatic Hydrolysis by Glu387Gly Enzyme. Anomerically ^{13}C-labeled 2,4-DNP-β-Glc (2 mg, 0.005 mmol), dissolved in 250 μL of 50 mM phosphate buffer, pH 6.5 in D_2O, was added to 30 μL (29.4 μg) of Glu387Gly enzyme preparations, before and after inhibition by 2,4-DNP-2F-β-Glc, and the reaction was followed by ^{13}C NMR spectroscopy for 20 min at room temperature. The spectra obtained did not show any substrate transformation. Sodium azide (37.5 mg, 0.57 mmol) or sodium formate (38.7 mg, 0.56 mmol) was dissolved in 200 μL of 50 mM sodium phosphate buffer, pH 6.5 in D_2O, and added to the reaction mixture, but the reaction did not start (as indicated by different ^{13}C NMR spectra taken during an additional 20 min of reaction time). After different cycles at 75 °C, the reaction started as registered by ^{13}C NMR spectra (see Figures 3 and 4 for details). Blank experiments, in which external nucleophiles or the biocatalysts were respectively absent, were performed in each case.

RESULTS AND DISCUSSION

Preparation of Active-Site Mutants. We have previously shown that the essential residue Glu387 of Ssβ-gly is the nucleophile of the reaction (*13*, *14*). In particular, the mutant Glu387Gln was completely inactive on all substrates tested. To elucidate the role of residue 387 in the reaction mechanism we have prepared two new mutants: Glu387Ala and Glu387Gly. Wild-type and mutant proteins were obtained as fusions with glutathione S-transferase (GST), as previously reported (*13*). This system allowed us to easily purify Ssβ-gly mutants with impaired enzymatic activity by taking advantage of GST activity. As previously reported, Ssβ-gly proteins obtained with this system contain a seven-residue extension at their N-terminus (*13*). No significant differences in behavior during purification could be detected and the far-UV circular dichroism spectrum of each mutant was indistinguishable from that of the wild-type enzyme, indicating that mutations did not affect the overall structure of the molecule (not shown).

Recovery of the Activity of the Ssβ-Gly Mutants Using External Nucleophiles. Enzyme activities of wild type and Glu387Ala, -Gly, and -Gln mutants were assayed at 65 °C on 2-NP-β-Glc, 4-NP-β-Glc, and 2,4-DNP-β-Glc. These substrates contain aryl groups that differ in their pK_a and therefore in their leaving ability. In particular, 2,4-dinitrophenol (pK_a 3.96, from ref *22*) is a better leaving group than 2- and 4-nitrophenol (pK_a 7.22 and 7.18, respectively). To correct for the spontaneous hydrolysis of substrates at high temperatures, controls were carefully performed throughout the following characterization by using a blank mixture containing all the reactants with the exception of the enzyme.

Extremely low activity on all substrates was recorded for the three mutants; when sodium azide or formate were included as external nucleophiles, a considerable rescue of the activity was obtained with Glu387Ala and Gly mutants on 2,4-DNP-β-Glc (Table 1). Remarkably, the Glu387Gly mutant showed increased activity, with the same nucleophiles, also on 2-NP-β-Glc; whereas its activity on 4-NP-β-Glc substrate did not change upon addition of external nucleophiles (see below for discussion).

The activity recovered by Glu387Ala and -Gly mutants with 2,4-DNP-β-Glc at 65 °C was dependent on the azide up to 3 M final concentration (data not shown). For all the following characterization sodium azide and sodium formate were used in the final concentration of 2 M.

Table 1: Effect of 2 M Sodium Azide and Sodium Formate on the Specific Activity of Wild-Type and Mutant Ssβ-Gly[a]

	wild type			Glu387Gln			Glu387Ala			Glu387Gly		
substrate	no addition	azide	formate	no addition	azide	formate	no addition	azide	formate	no addition	azide	formate
2-NP-β-Glc	59.1	60.2	67.0	0.2	0.1	0.1	0.7	1.1	0.9	0.7	2.0	5.6
4-NP-β-Glc	57.0	35.3	42.2	0.04	0.07	0.04	0.7	0.5	0.4	0.3	0.3	1.0
2,4-DNP-β-Glc	55.1	32.1	54	0.1	NA[b]	0.1	0.3	0.9	0.8	0.6	22.1	8.6

[a] Values are obtained from standard assays, are shown as units per milligram at 65 °C, and are corrected for the spontaneous rate observed in the absence of enzyme. [b] NA (no measurable activity) means that, with 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 approximately the same as in the control without enzyme.

Table 2: Analysis of Wild-Type Contamination in the Glu387Gly Mutant Preparation[a]

			Glu387Gly[c]			
			2-NP-β-Glc		4-NP-β-Glc	
	wild type[b]		−	+	−	+
	2-NP-β-Glc	4-NP-β-Glc	azide	azide	azide	azide
− inhibitor[d]	71.3	47.7	0.6	4.3/4.2	0.25	0.32/0.35
+ inhibitor	NA[e]	NA	NA	3.8/3.9	NA	0.11/0.05

[a] Values are obtained from standard assays, are shown as units per milligram at 65 °C, and are corrected for the spontaneous rate observed in the absence of enzyme. [b] The inhibitor/enzyme equivalent ratio used was 102:1. [c] Values separated by spheres refer to the two inhibitor/enzyme ratios used (100:1 and 0.6:1). [d] 2,4-Dinitrophenyl-2-deoxy-2-fluoro-β-glucoside. The assays were performed after overnight incubation at 50 °C. [e] NA (no measurable activity) means that the rates of change in absorbance did not vary in the experimental conditions and were approximately the same as in the control without enzyme.

No activity rescue could be obtained with the Glu387Gln mutant on all the substrates tested under the same conditions. It was inferred that this result might be due to the different nature of the substituting amino acid: the larger side chain of Gln, as compared with those of Ala and Gly, could not allow the external nucleophile to access the substrate in the active site.

Analysis and Inhibition of Residual Wild-Type Activity in the Gly Mutant Preparation. As the glutamic acid 387 is absolutely required for the reaction (*13*), the residual activity observed in the three mutants without nucleophiles (Table 1) was unexpected and raised the suspicion of contamination with small amounts of wild-type enzyme in the mutant preparations. For this reason, experiments with 2,4-dinitrophenyl-2-deoxy-2-fluoro-β-glucoside (2,4-DNP-2F-β-Glc), a mechanism-based inhibitor, were performed. 2-Deoxy-2-fluoroglycosides have been successfully used for the inactivation and site-specific labeling of the active site nucleophile of several glycosyl hydrolases (*23*−*26*) but not Ssβ-gly.

To ascertain the inhibitory effect of 2,4-DNP-2F-β-Glc, the wild-type Ssβ-gly was incubated with a 102-fold molar excess of 2,4-DNP-2F-β-Glc at 50 °C. It was observed that inactivation was 80% after 30 min and complete after overnight incubation (Table 2). This inactivation is not attributed to thermal denaturation, since the enzyme incubated under the same conditions, except for the inhibitor, was fully active after 24 h (data not shown). When 2 M sodium azide was included in the reaction mixture, no activity rescue was observed (data not shown), suggesting that the external nucleophile could not reactivate the inhibited enzyme. This experiment indicates that activated 2-deoxy-2-fluoro-β-glucoside is an inhibitor of Ssβ-gly and it can be used to probe the presence of wild type in preparations of active-site nucleophile mutants.

Similar experiments were performed on the Glu387Gly mutant. Incubation at 50 °C overnight, at two different equivalents of 2,4-DNP-2F-β-Glc per equivalent of enzyme ratios (100:1 and 0.6:1, respectively), gave 100% inactivation on both 2- and 4-NP-β-Glc substrates (Table 2). Nevertheless, when 2 M sodium azide was included in the reaction mixture, the activity of Glu387Gly was again recovered and no differences were observed at the two inactivator/enzyme equivalent ratios used (Table 2). This clearly indicates that the Glu387Gly mutant enzyme is insensitive to the 2,4-DNP-2F-β-Glc inhibitor and that it is the only factor responsible for the activity rescue observed in the presence of external nucleophiles. In fact, as described above, azide cannot recover the inhibited wild-type activity.

These results clearly indicate that the low activity observed in the Glu387Gly mutant preparation (Table 1) was due to contamination of small amounts of wild-type enzyme. It is possible that the low activity observed in Glu387Ala and -Gln mutants preparations is due to the contamination of wild type as well. The origin of the wild-type contamination is still obscure and probably occurred during the mutant enzyme preparation. However, it was not considered relevant for the purpose of this work (see also below).

Thermal Activity of the Ssβ-Gly Mutants. The dependence of the activity on temperature was investigated following the wild-type and the -Gly mutant activities within the range of 30−75 °C on 2,4-DNP-β-Glc and 2-NP-β-Glc with sodium azide and sodium formate (Figure 1). These experiments show that the specific activity of the mutant increases with temperature up to 75 °C. Hence, the enzyme maintains its thermophilicity even when the Glu387 nucleophile is replaced by external anions. With the 2-NP-β-Glc substrate, straight lines were observed from the Arrhenius plot, but the Q_{10} of the reaction catalyzed by Glu387Gly mutant was lower than that of the wild type (Figure 1a). The activation energies (E_a) calculated for this substrate are 57.3 kJ mol^{-1} for the wild type and 35.4 and 39.4 kJ mol^{-1} for the mutant in the presence of sodium azide and sodium formate, respectively. The enthalpies of activation ($\Delta H^{\ddagger}$), calculated from the E_a at 65 °C, were lower for the mutant (32.6 and 36.6 kJ mol^{-1} for azide and formate, respectively) than the wild type (54.5 kJ mol^{-1}), confirming that the reaction catalyzed by the mutant depended less on temperature. However, the $\Delta G^{\ddagger}$ values, which represent the true height of the energy barrier of the reaction, were similar for the wild type (364 kJ mol^{-1}) and the mutant in the presence of azide and formate (372 and 369 kJ mol^{-1}, respectively). With 2,4-DNP-β-Glc, a discontinuity of the slope around 55 °C in the Arrhenius plot of the Gly mutant in the presence of sodium azide was observed even upon increasing the substrate concentration up to 10 mM (Figure 1b). By

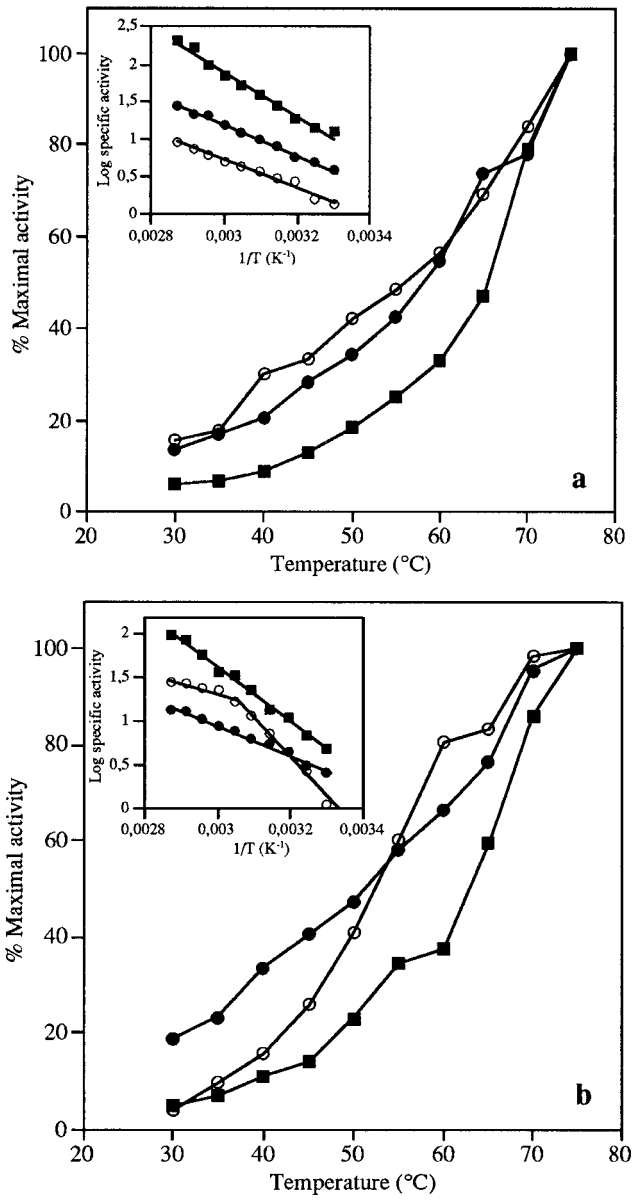


FIGURE 1: Dependence on temperature of the wild-type (■) and Glu387Gly mutant Ssβ-gly in the presence of 2 M sodium azide (○) and sodium formate (●), respectively. (a) 2-NP-β-Glc; (b) 2,4-DNP-β-Glc. Arrhenius plots are also shown (insets).

contrast, a straight line was obtained from the Arrhenius plot for the -Gly mutant with sodium formate and for the wild-type Ssβ-gly. The activation energies calculated were 32.9 and 59.9 kJ mol^{-1} for the mutant and the wild type, respectively. These values were comparable to those shown with the 2-NP-β-Glc substrate. Several explanations of the discontinuity observed with 2,4-DNP-β-Glc substrate can be proposed, i.e., the reaction catalyzed by the Gly mutant in the presence of azide could involve steps with different temperature coefficients. However, further experiments are in progress in order to better elucidate this behavior.

Kinetic Characterization of the Ssβ-Gly Mutants. The kinetic constants for hydrolysis at 65 °C shown by wild-type and mutant Ssβ-gly for 2,4-DNP-β-Glc and 2-NP-β-Glc substrates are reported in Tables 3 and 4, respectively. The maximal reactivation was observed with the Glu387Gly mutant on 2,4-DNP-β-Glc, whose specific activity was 40% and 15% with azide and formate, respectively, if compared

Table 3: Kinetic Constants for 2,4-DNP-β-Glc Hydrolysis at 65 °C of Wild-Type and Glu387Ala/Gly Mutant Ssβ-Gly[a]

enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
wild type	0.17 ± 0.04	275 ± 16	1617
wild type + 2 M azide	1.64 ± 0.20	428 ± 20	261
wild type + 2 M formate	0.61 ± 0.06	367 ± 12	602
Glu387Ala	NA[b]	NA	
Glu387Ala + 2 M azide	1.21 ± 0.12	9.6 ± 0.3	8
Glu387Gly	NA	NA	
Glu387Gly + 2 M azide	0.18 ± 0.03	110 ± 4.7	611
Glu387Gly + 2 M formate	0.13 ± 0.02	42 ± 1.2	323

[a] At least part of these activities appears to be due to contaminating wild-type enzyme (see text for details). Values are corrected for the spontaneous rate observed in the absence of enzyme. [b] NA (no measurable activity); see Table 1.

Table 4: Kinetic Constant for 2-NP-β-Glc Hydrolysis at 65 °C of Wild-Type and Glu387Gly Mutant Ssβ-Gly[a]

enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
wild type	1.01 ± 0.24	538 ± 11	533
wild type + 2 M azide	0.98 ± 0.10	480 ± 13	490
wild type + 2 M formate	0.50 ± 0.07	425 ± 11	850
Glu387Gly	NA[b]	NA	
Glu387Gly + 2 M azide	0.80 ± 0.11	15 ± 0.5	19
Glu387Gly + 2 M formate	1.17 ± 0.12	53 ± 1.2	45

[a] At least part of these activities appears to be due to contaminating wild-type enzyme (see text for details). Values are corrected for the spontaneous rate observed in the absence of enzyme. [b] NA (no measurable activity); see Table 1.

to the wild-type enzyme without nucleophiles. The affinity to the substrate appeared unchanged upon mutation, whereas the specificity constants with azide and formate were 38% and 20%, respectively, compared to the wild-type value. In the case of the -Ala mutant, the calculated k_{cat} and k_{cat}/K_M values for the same substrate, with 2 M sodium azide, were 3.5% and 0.5% those of the wild type, respectively. Both the catalytic efficiency and the reaction rate enhancements on 2,4-DNP-β-Glc observed with the two mutants, and particularly with Glu387Gly, are remarkably high if compared with the values for mesophilic glycohydrolases.[2] Peculiarly, significant reactivation of the Glu387Gly mutant on 2-NP-β-Glc, which has a relatively weak leaving group, was observed. Also in this case, the affinity to the substrate did not change upon mutation, but the catalytic efficiency was 3.5% and 8.4% with azide and formate, respectively, if compared to the wild type without nucleophiles (Table 4). The leaving ability of the two nitrophenol groups of the substrates and the nature of the anions appeared to affect in some way the efficiency of reactivation. In the presence of azide the specific activity of the Gly mutant on 2-NP-β-Glc was almost 10 times lower than on 2,4-DNP-β-Glc. However, in the presence of formate, the k_{cat} values for the two substrates were very similar.

[2] Reactivation of two mesophilic glycosyl hydrolases by external nucleophiles under similar conditions has been reported (*7*, *8*). In the case of the *Agrobacterium* β-glucosidase Glu358Ala mutant, only 1.2% and 3.4% of the wild-type specific activity was obtained with 2 M azide and formate, respectively. For the *Cellulomonas fimi* β-1,4-glycanase Glu233Ala mutant, the activity restored in the presence of 2 M azide and formate was only 0.2% and 0.6%, respectively. Moreover, reactivation of these mutants were obtained only with 2,4-DNP-β-glycosides.

The kinetic constants of the native Ssβ-gly were affected to some extent by external anions. The affinity and the specificity constants for 2,4-DNP-β-Glc substrate were reduced, whereas on 2-NP-β-Glc, formate produced a slight increment of the k_{cat}/K_M value (Tables 3 and 4).

None of the mutants is active on 4-NP-β-Glc substrate, although it contains an aglycon group with a pK_a similar to that of 2-NP-β-Glc. However, there is ample spectroscopic evidence that 2-nitrophenol can form a chelate ring by H-bonding: this may increase the leaving ability of this group upon protonation (*27*). Additional substrate properties with respect to the active-site interaction, other than the overall leaving group ability, such as the position of the nitro group on the phenolate ring, could play a relevant role in the efficiency of reactivation of Ssβ-gly mutants.

Identification of the Products. To evaluate whether the reaction mechanism was affected by mutation, the analysis of the products of the reaction catalyzed by wild-type and mutant Ssβ-gly was performed by ^{13}C NMR spectroscopy. The observation of enzymatic catalysis by using ^{13}C-labeled substrates directly in the NMR tube is a convenient way to detect the products of the enzymatic transformation as well as any side product arising from the labeled part of the substrate molecule. The synthesis of anomeric ^{13}C-labeled 2,4-DNP-β-Glc was performed, as recently reported, by direct anomeric *O*-arylation in a straightforward way starting from [1-^{13}C]glucose and 1-fluoro-2,4-dinitrobenzene (*20*). Although the yield of isolated product is not any larger than 20%, the protection and deprotection steps are in this case not necessary.

To ascertain the feasibility of the technique and to obtain information about chemical shift values, the analysis was at first performed by using wild-type Ssβ-gly for hydrolysis of 2,4-DNP-β-Glc in the absence of external anions at room temperature. At the beginning of the reaction only the anomeric carbon signal of the substrate is detected in the ^{13}C NMR spectra at 102.08 ppm (Figure 2a). As the reaction proceeds (10 min), the signal due to β-glucose (97.83 ppm) arises (Figure 2b) followed by the α-glucose signal (Figure 2c). Almost complete enzymatic hydrolysis is clearly observed after 20 h (Figure 2d). The fact that β-glucose is the first compound formed during the hydrolysis of 2,4-DNP-β-Glc and that the α-anomer appears later is a further direct indication of the *retaining* mechanism followed by this thermophilic enzyme in the hydrolysis reaction.

Reaction with Sodium Azide. Encouraged by this result, we followed a similar procedure in the reaction catalyzed by Glu387Gly mutant enzyme in the presence of sodium azide. The spectrum observed after 20 min of incubation at room temperature was similar to the one obtained at the beginning. This meant that the reaction did not proceed without heating. After 10 min at 75 °C the spectrum indicated the presence of a signal at 90.79 ppm due to α-glucosyl azide (Figure 3). The identity of this signal was established by preparing this product as reported in Experimental Procedures. This spectrum also demonstrated that the reaction mixture did not contain any anomeric form of glucose, and this led to the conclusion that Glu387Gly enzyme performs the hydrolysis of the substrate at high temperatures in the presence of sodium azide as an *inverting* glycosidase. Furthermore, the absence, even at the reaction temperature of 75 °C, of any signal due to β-glucosyl azide (91.40 ppm) arising from wild type indicates that the contamination occurring in the Glu387Gly preparation used for the experiments is negligible. To rule out the possibility of a chemical formation of α-glucosyl azide by nucleophilic displacement of the good leaving group 2,4-dinitrophenolate, a blank experiment was conducted in the absence of enzyme. In the early stages of the reaction no α-glucosyl azide was

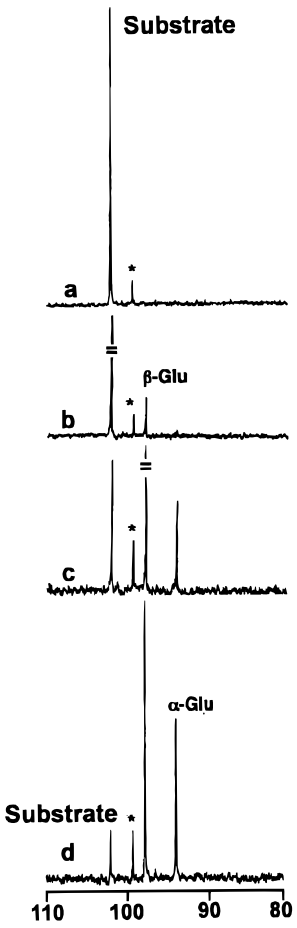


FIGURE 2: Enzymatic hydrolysis by wild-type Ssβ-gly of anomerically ^{13}C-labeled 2,4-dinitrophenyl-β-D-glucoside. The reaction was performed directly in the NMR tube at room temperature as described in the Experimental Procedures. The letters indicate the reaction time: a = 0, b = 10 min, c = 240 min, d = 20 h. The signal indicated by an asterisk is probably due to the α-anomer of the substrate purified by preparative TLC.

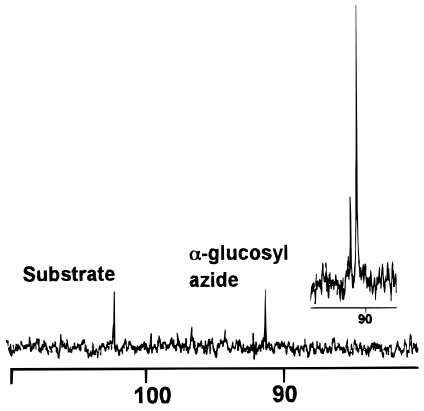


FIGURE 3: Enzymatic reaction performed by Glu387Gly mutant for 10 min at 75 °C in the presence of sodium azide. As a control, the spectrum of a 3:1 mixture of α- and β-glucosyl azides, respectively, is also shown.

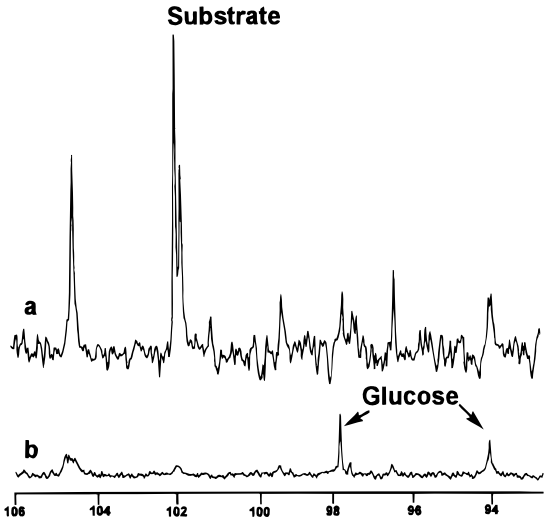


FIGURE 4: Enzymatic reaction performed by Glu387Gly mutant in the presence of sodium formate. (a) Spectrum run after 3 × 5 min cycles at 75 °C; (b) spectrum run after the addition of a total of 80 μL of wild-type enzyme (2.67 mg/mL) and 45 min reaction time.

formed. After 10 min formation was observed at an extent much lower than in the enzymatic reaction as calculated by substrate/product signal ratio (data not shown).

These findings indicate that the reaction catalyzed by the Glu387Gly mutant must proceed via direct attack of the azide to the α-side of the C1 of the substrate producing α-glucosyl azide (Scheme 1C). Interestingly, the azide attack is extremely selective since no β-glucosyl azide product could be detected by ^{13}C NMR spectroscopy experiments with the mutants. By contrast, the wild-type enzyme, when assayed in the presence of azide, followed the mechanism showed in Scheme 1B: in this case the small anion competes with water in the second step of the reaction and forms β-glucosyl azide by glycosyl transfer reaction.

Reaction with Sodium Formate. In the reaction of Glu387Gly with sodium formate, two interesting signals at 104.69 and 101.91 ppm originated from the anomeric carbon of the substrate after a 15 min reaction time (Figure 4a). The two signals did not appear in two blank experiments where sodium formate or the biocatalyst were respectively absent. An experiment on analytical scale starting with 46 μmol (16 mg) of unlabeled substrate was then performed for the identification of the reaction product. After purification of the reaction mixture by column chromatography and by TLC, a compound was isolated whose ^{1}H NMR spectrum showed two anomeric signals at δ 5.40 and 4.78, both having $J_{H1-H2} = 7-8$ Hz characteristic of β-linkage. Anomeric carbon signals in the ^{13}C NMR spectrum are in agreement as they are found at 104.7 and 101.9 ppm, the latter being easily assigned to internal sugar unit by comparing it with that of 2,4-DNP-β-Glc found at 102.01. Interglycosidic linkage assignment was based on the glycosylation shift rule (*12*, *28*) as observed for C3 (α-shift, 85.5 ppm) and C2, C4 (β shifts, 74.2 and 69.4 ppm) by comparison with signals of 2,4-DNP-β-Glc. These results, together with FAB-MS spectra of peracetylated derivative, indicate that the compound is a disaccharide derivative of 2,4-dinitrophenol in which the additional glucose molecule was β-linked to the 3-O-position of glucose.

The experiment described in Figure 4 was performed by using a preparation of mutant enzyme in which the wild-type activity has been inhibited by overnight treatment with 2,4-DNP-2F-β-Glc. This ruled out the possibility of wild-type-directed formation of the disaccharide molecule. Interestingly, the disaccharide formed by the mutant survives up to 15 min at 75 °C in the reaction mixture, indicating that it is recognized by the mutant neither as a substrate acceptor (no other signal for a conceivable trisaccharide synthesis could be detected) nor as substrate for the hydrolysis.[3] By contrast, the disaccharide molecule was consumed on addition of external wild-type enzyme to the reaction mixture and incubation at room temperature. Spectra were taken at 5 min intervals, showing a broadening of the signals at 104.69 and 101.91 (Figure 4b). This result is attributable to a transglycosylation operated by the wild-type enzyme forming different β-linked disaccharide derivatives. These results, and the rapid disappearance of the disaccharide observed with mutant preparation without the previous inhibition of wild-type contaminant (spectra not shown), further confirmed the action of the mutant in the disaccharide synthesis. Preliminary results indicate that a 3-*O*-β-linked disaccharide derivative of the substrate was also produced when the mutant reacted with sodium formate and 2-NP-β-Glc as substrate. Remarkably, by replacing the catalytic nucleophile, the regioselectivity of the reaction toward saccharide positions both changed and increased (only 3-*O*-products were formed). On the contrary, the wild-type enzyme showed moderate selectivity toward the 6-*O*-position of glucose-based acceptors (*12*).

The role of sodium formate in the reaction catalyzed by Ssβ-gly mutant appears different from the role of azide described above. In the presence of formate, the anomeric configuration of the product indicates that the Glu387Gly behaves as a *retaining* enzyme. This is the first evidence of this kind since no product characterization in the reaction with formate as external agent was reported for mesophilic enzymes (*7*, *8*). The restoration of the *retaining* reaction implies a two-step mechanism. The formate would participate in the first step, assisting the leaving of the phenolate group and then stabilizing the glucosyl unit, until it was transferred to a new substrate molecule. The role of sodium formate in the enzyme reactivation could be described as assistant nucleophile, with the formation of an intermediate state of unknown chemical nature (Scheme 1D). This role cannot be played by the azide, a strong nucleophile (*30*); thus the reaction stops after the first step, producing the stable α-product.

These results indicate that sodium formate has the optimal nucleophilicity for acting as a biomimicking agent since it is capable of restoring the function of the carboxylate group of Glu387 removed by mutation, and therefore it makes the synthesis of β-products possible. In contrast, the function of the azide is that of true nucleophile forming the covalent α-product.

[3] This resistance to hydrolysis can be explained by considering that Ssβ-gly shows exoglucosidase activity (*11*) with sugar-binding subsites $-1 + n$ ($1 < n < 5$), with substrate cleavage occurring between -1 and $+1$ (*29*). Hence, the β1−3 link of the disaccharide derivative cannot be hydrolyzed by the mutant since the 2,4-DNP-β-Glc would be a leaving group too weak for the nucleophilic attack of the formate.

CONCLUSIONS

The experiments described clearly showed that completely inactive mutants in the nucleophile residue of the *retaining* hyperthermophilic Ssβ-gly can be reactivated by the addition of external nucleophiles. Furthermore, the mechanism of the catalyzed reaction depends on the nature of the nucleophile added: in the presence of azide the mutant enzymes gave rise to product with *inverted* configuration (α-glycosyl azide), whereas in the presence of formate the observed product was a β-disaccharide *retaining* the configuration of the substrate. Remarkably, the product yielded by the reactivation with formate cannot be hydrolyzed by the mutant. Hence, this transglycosylation pathway (Scheme 1D) has potential application for the synthesis of disaccharides by exploiting this mutant as a thermophilic *glycosynthase*. A similar approach has been recently reported with an *Agrobacterium* β-glucosidase mutant and α-glycosyl fluoride as substrate (*31*).

ACKNOWLEDGMENT

We thank Dr. S. G. Withers for kindly providing 2,4-DNP-β-Glc substrates used for the preliminary experiments described in this work, the staff of ICMIB-CNR NMR service (S. Zambardino and V. Mirra) for NMR spectra, and Mr. Giovanni Imperato and Mr. Ottavio Piedimonte for technical assistance.

REFERENCES

1. McCarter, J. D., and Withers, S. G. (1994) *Curr. Opin. Struct. Biol. 4*, 885−892.
2. Davies, G., and Henrissat, B. (1995) *Structure 3*, 853−859.
3. Henrissat, B., and Bairoch, A. (1996) *Biochem. J. 316*, 695−696.
4. Koshland, D. E. (1953) *Biol. Rev. 28*, 416−436.
5. White, A., and Rose, D. R. (1997) *Curr. Opin. Struct. Biol. 7*, 645−651.
6. Kuroki, R., Weaver, L. H., and Matthews B. W. (1995) *Nat. Struct. Biol. 2*, 1007−1011.
7. Wang, Q., Graham, R. W., Trimbur, D., Warren, R. A. J., and Withers, S. G. (1994) *J. Am. Chem. Soc. 116*, 11594−11595.
8. MacLeod, A., Tull, D., Rupitz, K., Warren, R. A. J., and Withers S. G. (1996) *Biochemistry 35*, 13165−13172.
9. Moracci, M., Nucci, R., Febbraio, F., Vaccaro, C., Vespa, N., La Cara, F., and Rossi, M. (1995) *Enzyme Microb. Technol. 17*, 992−997.
10. Jenkins, J., Lo Leggio, L., Harris, G., and Pickersgill, R. (1995) *FEBS Lett. 362*, 281−285.
11. Nucci, R., Moracci, M., Vaccaro, C., Vespa, N., and Rossi, M. (1993) *Biotechnol. Appl. Biochem. 17*, 239−250.
12. Trincone, A., Improta, R., Nucci, R., Rossi, M., and Gambacorta, A. (1994) *Biocatalysis 10*, 195−210.
13. Moracci, M., Capalbo, L., Ciaramella, M., and Rossi, M. (1996) *Protein Eng. 9*, 1191−1195.
14. Febbraio, F., Barone, R., D'Auria, S., Rossi, M., Nucci, R., Piccialli, G., De Napoli, L., Orrù, S., and Pucci, C. (1997) *Biochemistry 36*, 3068−3075.
15. Aguilar, C., Sanderson, I., Moracci, M., Ciaramella, M., Nucci, R., Rossi, M., and Pearl, L. H. (1997) *J. Mol. Biol. 271*, 789−802.
16. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
17. Mikaelian, I., and Sergeant, A. (1992) *Nucleic Acids Res. 20*, 376.
18. Kuipers, O. P., Boot, H. J., and de Vos, W. M. (1991) *Nucleic Acids Res. 19*, 4558.
19. Leatherbarrow, R. J. (1992) *GraFit*, version 3.0, Erithacus Software Ltd., Staines, U.K.
20. Sharma, S. K., Corrales, G., and Penadés, S. (1995) *Tetrahedron Lett. 36*, 5627−5630.
21. Takeda, T., Sugiura, Y., Hamada, C., Fujii, L., Suzuki, K., Ogihara, Y., and Shibata, S. (1981) *Chem. Pharm. Bull. 29*, 3196−3201.
22. Kempton, J. B., and Whiters, S. G. (1992) *Biochemistry 31*, 9961−9969.
23. Withers, S. G., Warren, R. A. J., Street, I. P., Rupitz, K., Kempton, J. B., and Aebersold, R. (1990) *J. Am. Chem. Soc. 112*, 5887−5889.
24. Gebler, J. C., Aebersold, R., and Withers, S. G. (1992) *J. Biol. Chem. 267*, 11126−11130.
25. Miao, S., McCarter, J. D., Grace, M., Grabowski, G., Aebersold, R., and Withers, S. G. (1994) *J. Biol. Chem. 269*, 10975−10978.
26. Tull, D., and Withers, S. G. (1994) *Biochemistry 33*, 6363−6370.
27. Rao, C. N. R. (1969) in *Chemistry of nitro- and nitroso groups*, Part I, pp 112−116; Interscience, London.
28. Agrawal, P. K. (1992) *Phytochemistry 31*, 3307−3330.
29. Davies, G., Wilson, K. S., and Henrissat, B. (1997) *Biochem. J. 321*, 557−559.
30. Bunton, C. A. (1963) in *Nucleophilic substitution at a saturated carbon atom*, Elsevier Publishing Company, Amsterdam.
31. Mackenzie, L. F., Wang, Q., Warren, R. A. J., and Withers, S. G. (1998) *J. Am. Chem. Soc. 120*, 5883−5884.

BI981855F