Probing the Mechanism of *Bacillus* 1,3-1,4-β-D-Glucan 4-Glucanohydrolases by Chemical Rescue of Inactive Mutants at Catalytically Essential Residues†

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ABSTRACT: The role of the key catalytic residues Glu134 and Glu138 in the retaining 1,3-1,4-β-glucanase from *Bacillus licheniformis* is probed by a chemical rescue methodology based on enzyme activation of inactive mutants by the action of added nucleophiles. While Glu134 was proposed as the catalytic nucleophile on the basis of affinity labeling experiments, no functional proof supported the assignment of Glu138 as the general acid−base catalyst. Alanine replacements are prepared by site-directed mutagenesis to produce the inactive E138A and E134A mutants. Addition of azide reactivates the mutants in a concentration-dependent manner using an activated 2,4-dinitrophenyl glycoside substrate. The chemical rescue operates by a different mechanism depending on the mutant as deduced from ^{1}H NMR monitoring and kinetic analysis of enzyme reactivation. E138A yields the β-glycosyl azide product arising from nucleophilic attack of azide on the glycosyl−enzyme intermediate, thus proving that Glu138 is the general acid−base residue. Azide activates the deglycosylation step (increasing k_{cat}), but it also has a large effect on a previous step (as seen by the large decrease in K_M, the increase in k_{cat}/K_M, and the pH dependence of activation), probably increasing the rate of glycosylation through Brønsted acid catalysis by enzyme-bound HN_3. By contrast, azide reactivates the E134A mutant through a single inverting displacement to give the α-glycosyl azide product, consistent with Glu134 being the catalytic nucleophile. Formate as an exogenous nucleophile has no effect on the E138A mutant, whereas it is a better activator of E134A than azide. Although the reaction yields the normal hydrolysis product, a transient compound was detected by ^{1}H NMR, tentatively assigned to the α-glycosyl formate adduct. This is the first case where a nonmodified sugar gives a long-lived covalent intermediate that mimics the proposed glycosyl−enzyme intermediate of retaining glycosidases.

Glycosidases fall into two major mechanistic categories according to the stereochemical outcome of the bond cleavage: inverting enzymes, which hydrolyze the glycosidic bond with inversion of configuration, and retaining enzymes, which do so with net retention of the anomeric configuration (*1*, *2*). As far as the current evidence, both types of enzymes operate by general acid−base catalysis [where Asp, Glu, and Tyr have been identified as the catalytic residues (*3*)], but their mechanisms are distinctly different as a consequence of the active site topology (*2*, *4*). Inverting glycosidases have the catalytic residues, general acid and general base, ≈10 Å apart from each other, and they function through a single-step mechanism in which a water molecule effects a direct displacement at the anomeric center. Retaining enzymes, by contrast, function through a double-displacement mechanism via formation and hydrolysis of a glycosyl−enzyme intermediate, as originally proposed by Koshland (*5*). In the later case, the essential amino acid residues, the general acid−base and the catalytic nucleophile, are closer to each other at ≈5.5 Å.

Identification of the key essential residues is often approached by sequence comparison to other related enzymes that belong to the same family (*6*), but later the candidates must be probed experimentally. For retaining glycosidases, a number of methodologies have been applied, involving site-directed mutagenesis, affinity labeling or mechanism-based inactivation, and X-ray crystallography of enzyme−substrate or enzyme−inhibitor complexes. Withers and co-workers have developed an elegant approach for identifying the active site nucleophile by trapping the glycosyl−enzyme intermediate through the use of 2-deoxy-2-fluoro glycosides as mechanism-based inactivators (*7*−*9*). In contrast, techniques for the identification of the general acid−base residue are not so well developed (*9*), since affinity labeling may not be reliable. A promising approach that may become of general application is based upon a detailed kinetic analysis of mutants at the residue being the candidate functioning as the general acid−base catalyst, as nicely applied to a few retaining exoglycosidases (*10*−*12*).

In the framework of our structure−function studies of *Bacillus* 1,3-1,4-β-D-glucan 4-glucanohydrolases (EC 3.2.1.73, 1,3-1,4-β-glucanases[1]), this work describes mechanistic evidence from kinetic studies of mutants in probing the role

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of the catalytically essential residues previously identified by site-directed mutagenesis.

1,3-1,4-β-Glucanase is an endoglycosidase that is able to depolymerize mixed-linked glucans containing β-1,3- and β-1,4-glycosidic bonds such as barley β-glucan and lichenan, but it does not hydrolyze cellulose or laminarin derivatives. Cleavage of the natural substrates occurs regiospecifically on β-1,4-glycosidic linkages in 3-O-substituted glucopyranose units, as deduced from the structures of the final reaction products (*13*−*15*). Genes encoding bacterial 1,3-1,4-β-glucanases have been cloned from different *Bacillus* species, *Fibrobacter succinogenes*, *Ruminococcus flavofaciens*, and *Clostridium thermocellum* (*16*). All these enzymes share a high degree of sequence similarities, and they have been classified as members of family 16 glycosyl hydrolases (*4*, *6*, *17*, *18*). The three-dimensional structures of two wild type 1,3-1,4-β-glucanases from *Bacillus* [*Bacillus licheniformis* (*19*) at 0.18 nm resolution and *Bacillus macerans* (*20*)-at 0.23 nm resolution], as well as several hybrids of *Bacillus amyloliquefaciens* and *B. macerans* (*21*), have been solved by X-ray crystallography.

The *B. licheniformis* enzyme has been kinetically characterized using a new family of chromogenic low-molecular weight oligosaccharides with the general structure [Glcpβ-(1→4)]$_n$Glcpβ(1→3)Glcpβ-aryl (n = 0−3, aryl = 4-methylumbelliferyl, a series of substituted phenyl aglycons) (*22*, *23*). These substrates have the basic core substructure -4G3G-X according to the requirements of natural polysaccharide substrates (barley β-glucan and lichenan) for hydrolysis by the enzyme on a single glycosidic bond with release of the chromophoric aglycon. Kinetic studies have shown that the *B. licheniformis* enzyme is a retaining glycosidase (*15*) which has four glucopyranose-binding subsites on the nonreducing end from the scissile glycosidic bond, with subsite −III making the larger contribution to transition-state stabilization (*22*). Molecular modeling also suggests two or three subsites on the reducing end of the cleft (*24*). Because of the almost identical three-dimensional structures of *Bacillus* 1,3-1,4-β-glucanases, the biochemical and mechanistic results obtained for the *B. licheniformis* enzyme may be assumed to hold for all family 16 *Bacillus* isozymes.

Catalytic residues were identified by scanning mutagenesis where all the Asp and Glu residues that are conserved among the *Bacillus* 1,3-1,4-β-glucanases were replaced by the isosteric Asn and Gln residues. It was concluded that Glu138 and Glu134 are the key catalytic residues in the *B. licheniformis* enzyme (*25*, *26*). Likewise, the same residues were also identified in the *B. macerans* isozyme (*20*). Affinity labeling experiments on the *B. amyloliquefaciens* isozyme (*27*), and on a hybrid *B. amyloliquefaciens*−*B. macerans* (*28*), suggested the residue equivalent to Glu134 is the catalytic nucleophile. However, as will be argued in the Discussion, the epoxyalkyl labeling is not conclusive evidence on its own, since both the general acid−base and the nucleophile can be derivatized by this type of affinity label (*29*). This evidence is further compromised by the fact that there is no functional proof for assigning the other essential residue, Glu138, as the general acid−base catalyst.

[1] Abbreviations: 1,3-1,4-β-glucanase, 1,3-1,4-β-D-glucan 4-glucanohydrolase; G4G3G-2,4DNP, 2,4-dinitrophenyl 3-*O*-β-cellobiosyl-β-D-glucopyranoside; G4G3G-MU, 4-methylumbelliferyl 3-*O*-β-cellobiosyl-β-D-glucopyranoside; PCR, polymerase chain reaction.

Here we report on a chemical rescue methodology as an extension of the kinetic analysis approach first proposed by Withers and co-workers for identifying the general acid−base in exoglycosidases. For the *B. licheniformis* 1,3-1,4-β-glucanase, alanine replacements on both catalytic residues are prepared by site-directed mutagenesis to produce the inactive E138A and E134A mutants. Using a highly reactive substrate with a good leaving group such as the 2,4-dinitrophenyl glycoside, the activity may be restored by the action of an added reactive nucleophile. Both mutants are kinetically characterized and screened for the generation of new reaction products in the presence of exogenous nucleophiles.

MATERIALS AND METHODS

Reagents and Substrates. Restriction endonucleases and T4 DNA ligase were from Boehringer Mannheim, and Deep Vent polymerase was from New England Biolabs. DNA sequencing was performed using the femtomole sequencing kit from Pharmacia Biotech Inc. Oligonucleotides were synthesized by Gomensoro Biotech or Boehringer Mannheim. Growth media components were obtained from Scharlau. Barley β-glucan was from Megazyme, and calcium chloride, sodium azide, sodium formate, sodium acetate, and sodium propionate were from Sigma Chemical Co. 4-Methylumbellyferone and 2,4-dinitrophenol were obtained from Fluka, and they were recrystallized from glacial acetic acid (Sigma Chemical Co.). The substrates 4-methylumbellyferyl 3-*O*-β-cellobiosyl-β-D-glucopyranoside (G4G3G-MU) (*30*) and 2,4-dinitrophenyl 3-*O*-β-D-cellobiosyl-β-D-glucopyranoside (G4G3G-2,4DNP) (*23*) were synthesized as described previously. All buffers and solutions for kinetic experiments were filtered (0.45 μm) prior to being used.

Bacterial Strains and Culture Media. *Escherichia coli* TG1 [*supE hsd*Δ*5 thi* Δ(*lac-proAB*) *F*′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)] was used for plasmid propagation, transformation with the mutagenic polymerase chain reaction (PCR), and protein expression. Bacteria were grown in 2YT (16 g of bactotryptone, 10 g of yeast extract, and 5 g of sodium chloride in 1 L of H_2O) medium for plasmid isolation and in 2SB (24 g of bactotryptone, 48 g of yeast extract, 31.5 g of $Na_2HPO_4 \cdot 12H_2O$, and 1.7 g of KH_2PO_4 in 1 L of H_2O) medium for protein expression. Ampicillin at 100 μg mL^{-1} was added when it was appropriate.

Site-Directed Mutagenesis by PCR. The plasmid pUC119:: pD6-2 was used as a template for the mutagenic PCRs. It contains the gene coding for the *B. licheniformis* 1,3-1,4-β-glucanase, its constitutive promoter, signal peptide sequence, and terminator as a 1.21 kb *Sac*I−*Sph*I fragment (*25*). Site-directed mutagenesis was performed by the double PCR (*31*) as used previously for other 1,3-1,4-β-glucanase mutants (*26*, *32*, *33*). The mutagenic primers were as follows: E138A, 5′-GAT GAA ATC GAC ATC GCA TTT CTA GGA AAA GAT-3′; and E134A, 5′-CG CCT TGG GAT GCA ATC GAC A-3′. The mutagenic primer and the Forward Universal Primer flanking the 3′-end of the 1,3-1,4-β-glucanase gene are used in the first PCR. The amplified fragment and the Reverse Universal Primer are used in the second PCR to yield the whole 1,3-1,4-β-glucanase gene with the desired mutation. The final amplified DNA was cut with *Eco*RI−*Hin*dIII and ligated again into a pUC119 vector. After

transformation of *E. coli* TG1 cells, transformants were screened for inactive clones. It was done on replica plates of LB medium supplemented with 0.04% (w/v) barley β glucan, where the β-glucanase-active clones are detected as halo-forming colonies by the Congo red staining assay (*34*). After selection of three inactive clones for each mutagenesis reaction, the genes were full-length sequenced using appropriate primers located ~100 bases from the mutation point.

Expression and Purification of Wild Type (WT) and Mutant Enzymes. Proteins were purified from the supernatant of recombinant *E. coli* TG1 cultures harboring the wild type or mutagenized plasmids basically as described previously (*33*, *35*). The procedure involves two chromatographic steps after acid precipitation of most of the *E. coli* proteins. The first step is cation-exchange chromatography on CM-Sepharose (Pharmacia Biotech Inc.) in 5 mM sodium acetate buffer at pH 6.0, where proteins are eluted with a linear gradient of 0 to 0.4 M NaCl in the same buffer. Following dialysis and concentration, proteins were further purified by fast protein liquid chromatography on a cation-exchange TSK CM-3SW column in sodium acetate buffer at pH 5.5 and eluted with a linear gradient of 0 to 0.4 M NaCl in the same buffer. After dialysis against 2 mM MOPS at pH 7.0 (to raise the pH) and water, proteins were freeze-dried and lyophilized for storage. They were redissolved in 0.5 mM citrate/8 mM phosphate buffer at pH 7.2 for kinetic experiments. The purity was higher than 95% as judged by SDS−polyacrylamide gel electrophoresis according to Laemmli (*36*). Enzyme concentrations were determined by UV spectrophotometry using an ϵ_{280} of 14.5 mg^{-1} mL cm^{-1} (3.55×10^5 M^{-1} cm^{-1}) (*37*).

Enzyme Kinetics. *(a) Specific Activity with Barley β-Glucan*. Specific activity was determined by incubating a solution of 4 mg mL^{-1} barley β-glucan in 30 mM phosphate buffer at pH 7.0 and 0.1 mM $CaCl_2$ in a thermostated bath at 55 °C. After preincubation for 5 min, reactions were initiated by adding the enzyme to final concentrations of 4−6 nM for WT, 118 nM for E138A, and 574 nM for E134A. Initial rates were obtained by determining the net release of reducing sugars at 2 min intervals for a total time of 8 min by the Somogyi−Nelson method (*38*, *39*). Reducing power was expressed as equivalent glucose concentration, and specific activities are given in micromoles of glucose per minute per milligram.

(b) Kinetics with G4G3G-MU and G4G3G-DNP. All kinetics were determined by following changes in UV absorbance due to the release of 4-methylumbelliferone or 2,4-dinitrophenol using matched 1 cm path length cells in a Cary 4E spectrophotometer with a Peltier temperature control system which maintained the cells at 30 °C. Rates of the enzyme-catalyzed reactions were determined by incubating the enzyme at substrate concentrations ranging between $0.25K_M$ and $8.5K_M$ in citrate/phosphate buffer (6.5 mM citric acid and 87 mM Na_2HPO_4) at pH 7.2 and 0.1 mM $CaCl_2$ for 5−90 min in the thermostated cell holder. Reactions were initiated by addition of substrate to a preincubated mixture of buffer, calcium chloride, enzyme, and exogenous nucleophile and monitoring the absorbance change when λ = 365 nm for G4G3G-MU (release of 4-methylumbelliferone, $\Delta\epsilon_{365}$ = 5136 M^{-1} cm^{-1}) or when λ = 425 nm for G4G3G-2,4DNP (release of 2,4-dinitrophenol, $\Delta\epsilon_{425}$ = 6134 M^{-1} cm^{-1}; see below). For the E138A reaction with G4G3G-2,4DNP in the presence of sodium azide, K_M values drop to the micromolar range. The lowest substrate concentration assayed was 10 μM, and K_M values are only approximate in the range of 1−10 μM.

The concentrations of stock solutions of substrates were determined by UV spectrophotometry using the molar extinction coefficients measured at the wavelength of maximum absorbance (G4G3G-MU's ϵ_{316} = 12 699 M^{-1} cm^{-1} and G4G3G-2,4DNP's ϵ_{282} = 8690 M^{-1} cm^{-1}). The molar extinction coefficients at the working wavelengths were as follows: ϵ_{365} = 7 M^{-1} cm^{-1} for G4G3G-MU and ϵ_{425} = 24 M^{-1} cm^{-1} for G4G3G-2,4DNP. The pH-dependent molar extinction coefficient of 4-methylumbelliferone (MU-OH) at 365 nm and of 2,4-dinitrophenol (2,4DNP-OH) at 425 nm were accurately measured under the conditions used for the kinetic assays using stock solutions of both compounds (recrystallized three times from AcOH) in the appropriate buffer, the pH adjusted at 30 °C: ϵ_{365} = 5143 M^{-1} cm^{-1} for MU-OH and ϵ_{425} = 6158 M^{-1} cm^{-1} for 2,4DNP-OH.

^{1}H NMR Monitoring. ^{1}H NMR monitoring of enzymatic reactions was performed following a modification of the procedure reported by Malet et al. (*15*). ^{1}H NMR spectra were recorded on a Varian Gemini 300 spectrometer. Experiments were performed at 25 °C in D_2O at pD 7.3 (adjusted with 1 N NaOD/D_2O). Spectra were recorded at intervals of 5−60 min. Enzyme and nucleophile concentrations in experiments using the G4G3G-MU (8 mM) substrate were 10.2 μM WT and 1 M NaN_3, 12.3 μM E138A and 1 M NaN_3, and 9 μM E134A and 1 M NaN_3. Enzyme and nucleophile concentrations in experiments using the G4G3G-2,4DNP (10 mM) substrate were 12 nM WT, 0.40 μM E138A and 1 M NaN_3, 34.3 μM E134A and 2 M NaN_3, and 11.4 μM E134A and 2 M HCOONa.

RESULTS

Mutagenesis and Purification of Proteins. Alanine mutants at positions 134 and 138 were prepared by PCR site-directed mutagenesis. Transformants were screened for inactive mutants on plates supplemented with barley β-glucan and stained for activity. The efficiency of mutagenesis, based on the frequency of inactive transformants, was 91 and 76% for the E138A and E134A mutagenesis reactions, respectively. Expression levels and purification yields of the mutant proteins were similar to those reported for the wild type enzyme (*33*). They were homogeneous as judged by SDS−polyacrylamide gel electrophoresis and HPLC. Both mutants are essentially inactive toward the barley β-glucan substrate (specific activities in micromoles of glucose equivalents per minute per milligram, 900 ± 60 for WT, <0.2 for E138A, and <0.1 for E134A).

Substrates. On the basis of our previous kinetic studies on *Bacillus* 1,3-1,4-β-glucanases (*22*), the aryl oligosaccharides derived from 3-*O*-β-cellobiosyl-D-glucopyranose were chosen for this study. These trisaccharide derivatives fulfill the basic requirements of natural polysaccharides (barley β-glucan and lichenan) for hydrolysis by the enzyme, i.e., cleavage of a glycosidic bond on a 3-O-substituted glucopyranose unit. No internal glycosidic bonds are hydrolyzed, and only the chromophoric aglycon is released upon enzy-

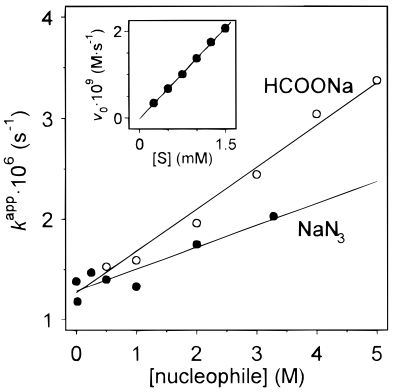
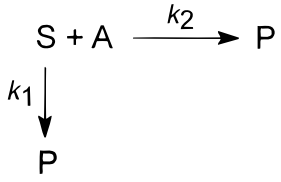


FIGURE 1: Kinetics of spontaneous (nonenzymatic) hydrolysis of G4G3G-2,4DNP in the presence of sodium azide and sodium formate in citrate/phosphate buffer and 0.1 mM $CaCl_2$ at pH 7.2 and 30 °C. k^{app}'s are the first-order rate constants of hydrolysis at different azide (●) and formate (○) concentrations ($k^{app} = k_2$[A] according to eq 1). (Inset) Spontaneous hydrolysis of G4G3G-2,4DNP in the absence of added nucleophiles (v_0 vs [G4G3G-2,4DNP]).

matic hydrolysis, allowing easy monitoring of the reaction course by continuous UV spectrophotometry. The 4-methylumbelliferyl glycoside (G4G3G-MU) has a poor leaving group (pK_a for 4-methylumbelliferone of 7.5), whereas the 2,4-dinitrophenyl glycoside (G4G3G-2,4DNP) is a highly reactive substrate with a good leaving group (pK_a for 2,4-dinitrophenol of 4.0). The synthesis of both substrates has recently been reported by our group with total synthesis (*30*) and with a chemoenzymatic approach (*23*, *40*).

Rate Constants of Spontaneous Substrate Hydrolysis. The 2,4-dinitrophenyl glycoside is readily hydrolyzed in solution at pH 7.2. To reduce the spontaneous hydrolysis in the enzymatic experiments, the temperature for kinetic measurements was decreased from 55 °C [temperature of maximum activity of the wild type enzyme (*25*)] to 30 °C. Spontaneous hydrolysis (nonenzymatic) follows first-order kinetics with a rate constant of $(13.8 \pm 0.07) \times 10^{-7}$ s^{-1} (Figure 1, inset). Exogenous nucleophiles increased the hydrolysis rate in a concentration-dependent manner. Rate constants were determined over the concentration range of nucleophiles used in the enzymatic studies (0−3.3 M azide and 0−5 M formate) by measuring the release of 2,4-dinitrophenol. Data in Figure 1 were fit to eq 1, which corresponds to the following model describing spontaneous (nonenzymatic) hydrolysis:



$$v = (k_1 + k_2[A])[S] \quad (1)$$

where A is the activator nucleophile (azide or formate) and S the 2,4-dinitrophenyl glycoside substrate. $k_1 = (12.9 \pm 0.07) \times 10^{-7}$ s^{-1} and $k_2 = (2.13 \pm 0.05) \times 10^{-7}$ M^{-1} s^{-1} for sodium azide as an activator, and $k_1 = (12.0 \pm 0.8) \times 10^{-7}$ s^{-1} and $k_2 = (4.4 \pm 0.3) \times 10^{-7}$ M^{-1} s^{-1} for sodium formate as an activator. Spontaneous hydrolysis of the 4-methylumbelliferyl glycoside was not detected under the same experimental conditions.

Enzyme Kinetics. Kinetics of the enzymatic reactions were determined in citrate/phosphate buffer at pH 7.2 and 30 °C. Initial velocities (<3% conversion) at different substrate concentrations were determined by monitoring the release of 4-methylumbelliferone when $\lambda = 365$ nm for G4G3G-MU ($\Delta\epsilon_{365} = 5136$ M^{-1} cm^{-1}) and 2,4-dinitrophenol when $\lambda = 425$ nm for G4G3G-2,4DNP ($\Delta\epsilon_{425} = 6134$ M^{-1} cm^{-1}). Spontaneous substrate hydrolysis rates were subtracted from the observed rates to obtain the enzyme-catalyzed velocities. Michaelis−Menten parameters for the WT and both alanine mutants are summarized in Table 1. Mutants are very inactive compared to the WT enzyme, necessitating great care in monitoring of initial reaction rates, and the K_M value of E134A with G4G3G-MU could not be determined. k_{cat}/K_M values for the two substrates are vastly different, that for the activated substrate being much higher than that for the other.

Effect of Exogenous Nucleophiles on Reaction Rates. Sodium salts of azide, formate, acetate, and propionate were used as exogenous nucleophiles in the enzyme-catalyzed hydrolysis of the two aryl glycoside substrates. While acetate and propionate had no effect, azide and formate behaved as inhibitors or activators depending on the enzyme and substrate (Table 2). The hydrolysis of both substrates by the WT enzyme is inhibited by azide and formate (Figure 2). Kinetic data were fit to competitive (eq 2) or mixed type (eq 3) inhibition models (*41*)

$$v_0 = \frac{V_{max}[S]}{[S] + K_M\left(1 + \frac{[I]}{K_I}\right)} \quad (2)$$

where I is azide or formate and S the substrate. K_I values

$$v_0 = \frac{V_{max}[S]}{[S]\left(1 + \frac{[I]}{\alpha K_I}\right) + K_M\left(1 + \frac{[I]}{K_I}\right)} \quad (3)$$

(Table 2) are very high, only reflecting the fact that both nucleophiles are poor reversible inhibitors. E134A and E138A mutants are also inhibited when using G4G3G-MU as the substrate. Due to the low activity of the mutants, the inhibition constants could not be accurately measured, and only IC_{50} values are reported (Table 2).

In contrast, the kinetic parameters of both mutants with the activated G4G3G-2,4DNP substrate were found to vary substantially with azide concentration. For E138A, k_{cat} increased up to 60-fold whereas K_M decreased drastically 65-fold in the presence of 1 M azide as shown in Figure 3. In terms of k_{cat}/K_M, the mutant is reactivated up to the wild type activity at 1 M azide (Table 1). In the case of E134A, both k_{cat} and K_M increased as a function of azide concentration. K_M leveled off at high concentrations (1−3.3 M azide), while k_{cat} showed an 80-fold increase at 3.3 M azide although did not reach saturation (Figure 4A). Activation of E138A by sodium azide was pH-dependent. Table 3 shows the steady-state velocities of the reaction of E138A with 1 mM G4G3G-2,4DNP (saturating) in the presence of 1 M NaN_3 at pH 6.4−8.3. The apparent reactivity of azide increases with decreasing pH.

Table 1: Kinetic Parameters for Wild Type and Mutant *B. licheniformis* 1,3-1,4-β-Glucanases in the Absence and Presence of Exogenous Nucleophiles[a]

enzyme	substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
WT	G4G3G-MU	0.67 ± 0.02	0.79 ± 0.04	850 ± 70
	G4G3G-2,4DNP	603 ± 8	0.20 ± 0.01	$(3.0 \pm 0.2) \times 10^6$
E138A	G4G3G-MU	$(6.7 \pm 0.4) \times 10^{-4}$	0.34 ± 0.07	2.0 ± 0.5
	G4G3G-2,4DNP	0.256 ± 0.005	0.22 ± 0.02	$(1.2 \pm 0.1) \times 10^3$
	with 1 M NaN_3	15.4 ± 0.4	≈ 0.005	$\approx 3.0 \times 10^6$
	with 4 M HCOONa	no reactivation		
E134A	G4G3G-MU	$(4 \pm 2) \times 10^{-6}$	nd	–
	G4G3G-2,4DNP	$(7.1 \pm 0.1) \times 10^{-4}$	0.16 ± 0.02	4.4 ± 0.5
	with 3.3 M NaN_3	$(5.6 \pm 0.2) \times 10^{-2}$	1.1 ± 0.7	$(5 \pm 1) \times 10^1$
	with 4 M HCOONa	2.10 ± 0.15	0.35 ± 0.04	$(6 \pm 1) \times 10^3$

[a] Conditions: 6.5 mM citrate/87 mM phosphate buffer at pH 7.2 and 30 °C, 0.1 mM $CaCl_2$, [G4G3G-MU] = 0.2−3 mM, and [G4G3G-2,4DNP] = 0.01−2 mM. Enzymes concentrations: 10 nM to 1 μM (E138A), 5 nM to 1 μM (E134A), and 0.1 nM to 0.14 μM (WT). Kinetic parameters at NaN_3 and HCOONa concentrations are given in Figures 3 and 4. nd, not determined.

Table 2: Effect of Exogenous Nucleophiles on Wild Type and Mutant 1,3-1,4-β-Glucanases

enzyme	substrate[a]	nucleophile	effect[b]	K_I (M)
WT	G4G3G-MU	azide	inhibition, mixed type ($\alpha = 2.2 \pm 0.2$)	0.33 ± 0.004
		formate	inhibition, mixed type ($\alpha = 0.74 \pm 0.02$)	1.3 ± 0.2
	G4G3G-2,4DNP	azide	inhibition, competitive	0.48 ± 0.15
		formate	inhibition, competitive	>5
E138A	G4G3G-MU	azide	inhibition	IC_{50}[c] ≈ 0.7 M
		formate	inhibition	$IC_{50} \approx 0.5$ M
	G4G3G-2,4DNP	azide	activation[d]	
		formate	no effect	
E134A	G4G3G-MU	azide	inhibition	$IC_{50} \approx 0.5$ M
		formate	inhibition	$IC_{50} \approx 1.0$ M
	G4G3G-2,4DNP	azide	activation[d]	
		formate	activation[d]	

[a] MU is 4-methylumbelliferyl, and 2,4DNP is 2,4-dinitrophenyl. [b] Conditions: 6.5 mM citrate/87 mM phosphate buffer at pH 7.2 and 30 °C, 0.1 mM $CaCl_2$, [G4G3G-MU] = 0.2−3 mM, [G4G3G-2,4DNP] = 0.1−2 mM, [azide] = 0−2 M, and [formate] = 0−4.5 M. [c] IC_{50} is the inhibitor concentration at which enzyme activity is decreased to 50%. [d] Addition of the exogenous nucleophile activates the enzyme (see the text).

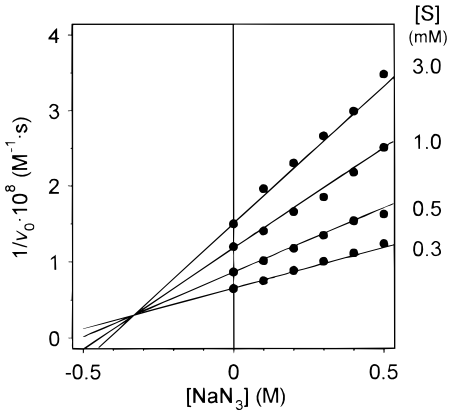


FIGURE 2: Dixon plot for the inhibition by sodium azide of the hydrolysis of G4G3G-MU by the wild type enzyme. Conditions were as follows: citrate/phosphate buffer and 0.1 mM $CaCl_2$ at pH 7.2 and 30 °C. Substrate and azide concentrations are given in the plot.

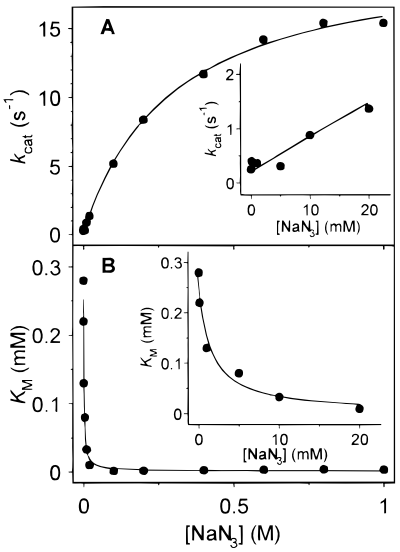


FIGURE 3: Kinetic parameters for hydrolysis of G4G3G-2,4DNP by the E138A mutant in the presence of various concentrations of sodium azide: (A) effect on k_{cat} and (B) effect on K_M. Insets are magnifications of the corresponding plots at low azide concentrations. Experimental conditions are given in Table 1.

When formate was used as an exogenous nucleophile in the enzyme-catalyzed reaction of the G4G3G-2,4DNP substrate, no effect was observed on E138A kinetics, whereas E134A showed a concentration-dependent reactivation (Figure 4B). k_{cat} did not reach saturation up to 4 M formate, but the magnitude of the activity (3000-fold increase) was much higher than that obtained with azide as an exogenous nucleophile (Table 1). K_M, on the other hand, only changed slightly, with a 2-fold increase relative to that of the nucleophile-free E134 mutant.

Effect of Exogenous Nucleophiles on Reaction Products. The enzymatic reactions were monitored by ^{1}H NMR

Table 3: Apparent Reactivity of E138A with G4G3G-2,4DNP in the Presence of Sodium Azide at Different pHs

pH	$v_0 \times 10^7$ (M s^{-1})[a]	pH	$v_0 \times 10^7$ (M s^{-1})
6.4	1.66	7.5	1.10
6.7	1.51	8.1	0.50
7.1	1.41	8.3	0.29

[a] Initial rates of 2,4-dinitrophenol release. Conditions: citrate/phosphate buffer and 0.1 mM $CaCl_2$ at 30 °C, [enzyme] = 10 nM, [G4G3G-2,4DNP] = 1 mM (saturating), and [NaN_3] = 1 M.

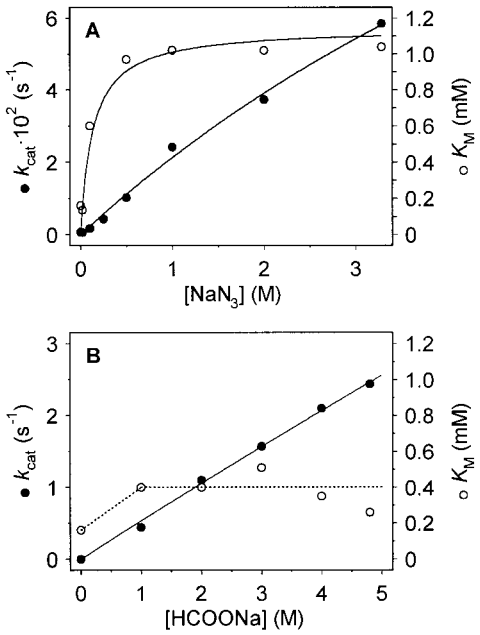


FIGURE 4: Kinetics of E134A reactivation by exogenous nucleophiles for the hydrolysis of G4G3G-2,4DNP: k_{cat} (●) and K_M (○) and (A) effect of sodium azide and (B) effect of sodium formate. Conditions are given in Table 1.

spectroscopy (in D_2O at pD 7.3 and 25 °C) for structure determination of the final products in the presence of exogenous nucleophiles. First, the wild type-catalyzed hydrolysis of G4G3G-2,4DNP was recorded as a reference. A doublet at 4.66 ppm (J = 8 Hz) is formed during the first minutes of reaction (Figure 5), proving the β-configuration on H-1A of the newly formed reducing sugar. Significant mutarotation is observed after reaction for only 20 min, slowing the apparent formation of reducing ends with the β-configuration and leading to the thermally equilibrated mixture of anomers. The NMR spectrum of the final product is identical to that previously reported for the hydrolysis of G4G3G-MU by the wild type enzyme (*15*, *22*). No change in the spectra was observed in the presence of added sodium azide, indicating that the exogenous nucleophile does not compete with the normal hydrolysis reaction.

As shown in Figure 6A, reaction of G4G3G-2,4DNP with E138A in the presence of 1 M sodium azide under the same experimental conditions yielded the β-glycosyl azide product. As main features in the time course monitoring, a doublet at 4.76 ppm (J = 8 Hz), which corresponds to the β-anomer, develops at the same rate at which the doublet corresponding to H-1A of the substrate (5.44 ppm, J = 7.5 Hz) disappears. No hydrolysis products are detected when the final spectrum is compared to that of the wild type enzyme reaction. On the other hand, time course monitoring of the reaction of the same substrate with E134A and 2 M sodium azide shows

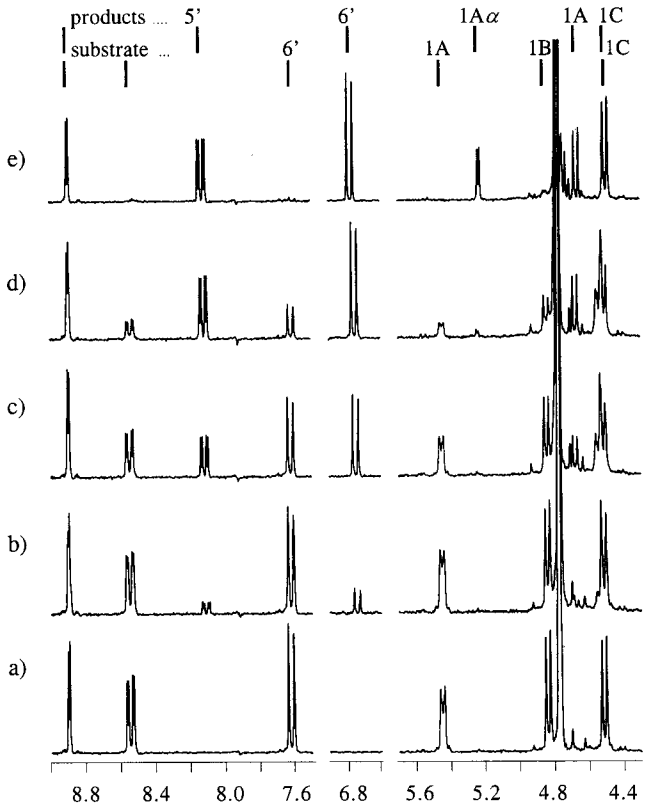


FIGURE 5: ^{1}H NMR spectra for the wild type-catalyzed hydrolysis of G4G3G-2,4DNP. The reaction was carried out in D_2O at 25 °C and pD 7.3. [E] = 12 nM, and [S] = 10 mM. (a) G4G3G-2,4DNP substrate at t = 0 and (b−e) after 3, 15, and 30 min and 24 h. δ values are given in parts per million (J in hertz): 4.48 (8.1) H-1C substrate, 4.49 (8.1) H-1C product, 4.66 (8.1) H-1A product (β-anomer), 4.76 (8.1) H-1B product, 4.81 (8.1) H-1B substrate, 5.23 (3.5) H-1A product (α-anomer), 5.44 (7.5) H-1A substrate, 6.72 (9.5) H-6′ 2,4-dinitrophenol, 7.61 (9.5) H-6′ substrate, 8.10 (3.0, 9.5) H-5′ 2,4-dinitrophenol, 8.54 (3.0, 9.5) H-5′ substrate, 8.90 (3.0) H-3′ substrate, 8.90 (3.0) H-3′ 2,4-dinitrophenol.

the formation of a doublet at 5.52 ppm (J = 3.6 Hz), which is assigned to the α-glycosyl azide product (Figure 6B). Again, no hydrolysis products are detected, and the final glycosyl azide is stable in the NMR tube. Both reactions are complete, no initial substrate being detected after long incubation times.

A different behavior was observed when sodium formate was used as the exogenous nucleophile with the E134A mutant. The final product of formate reactivation was the same hydrolysis product of the normal wild type reaction, the trisaccharide G4G3G (Figure 7). Both the β-anomer (4.66 ppm, J = 8 Hz) and the α-anomer (5.23 ppm, J = 3.5 Hz) are present in the reaction mixture after 22 h. However, a transient compound with a lifetime of approximately 7 h was detected, as seen by the new doublet at 5.40 ppm (J = 4.0 Hz). This compound was tentatively assigned to the transient α-glycosyl formate intermediate shown in Scheme 1. It is slowly hydrolyzed to yield the final reducing sugar, but it is not clear whether the β- or α-anomer of the hydrolysis product is formed first before mutarotation equilibrates the final mixture.

DISCUSSION

Enzyme Mechanism and Catalytic Residues. The mechanism of a retaining glycosidase involves a double-displace-

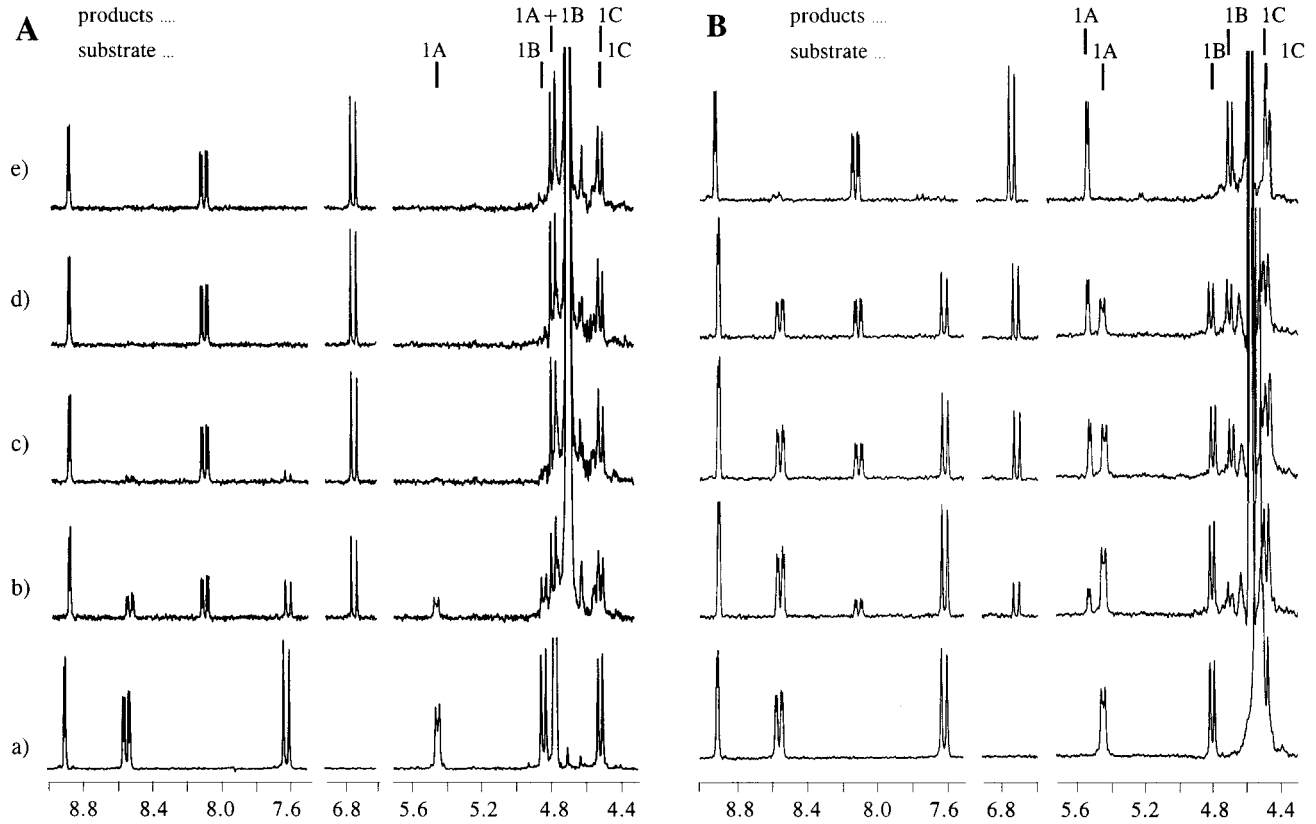


FIGURE 6: ^{1}H NMR monitoring of the reaction of G4G3G-2,4DNP with 1,3-1,4-β-glucanase mutants in the presence of sodium azide. The reaction was carried out in D_2O at 25 °C and pD 7.3. (A) E138A mutant where [E] = 0.4 μM, [S] = 10 mM, and [azide] = 1 M: (a) G4G3G-2,4DNP substrate at t = 0 and (b−e) after 5, 10, 15, and 20 min. δ values are given in parts per million (J in hertz): 4.48 (8.1) H-1C substrate, 4.49 (8.1) H-1C β-glycosyl azide, 4.76 (8.1) H-1A,1B β-glycosyl azide, 4.81 (8.1) H-1B substrate, 5.44 (7.5) H-1A substrate. (B) E134A mutant, where [E] = 34.3 μM, [S] = 10 mM, and [azide] = 2 M: (a) G4G3G-2,4DNP substrate at t = 0 and (b−e) after 1, 4, 7, and 45 h. δ values are given in parts per million (J in hertz): 4.48 (8.1) H-1C substrate, 4.49 (8.0) H-1C α-glycosyl azide, 4.70 (8.1) H-1B α-glycosyl azide, 4.81 (8.1) H-1B substrate, 5.44 (7.5) H-1A substrate, 5.52 (3.6) H-1A α-glycosyl azide. Assignment of the aromatic signals (6.6−9.0 ppm) is given in Figure 5.

ment reaction assisted by general acid−base catalysis (Scheme 2) (*1*−*3*). In the first step (glycosylation), the amino acid residue acting as a general acid protonates the glycosidic oxygen with concomitant C−O breaking of the scissile glycosidic bond, while the deprotonated carboxylate functioning as a nucleophile stabilizes the oxocarbenium intermediate through electrostatic interaction or by forming a covalent glycosyl−enzyme intermediate. Supporting evidence from trapping experiments using substrate analogues suggests that a covalent intermediate is more likely to be formed in most of the retaining glycosidases (*42, 43*). The second deglycosylation step involves the attack of a water molecule assisted by the conjugate base of the general acid which renders the free sugar with overall retention of configuration, and the enzyme returns to its initial protonation state.

The catalytic residues of *Bacillus* 1,3-1,4-β-glucanases have been identified by mutational analysis and X-ray crystallography, but the functional role of each residue is not conclusive. Identification of E134 as the catalytic nucleophile in the *B. licheniformis* enzyme was supported by affinity labeling experiments on the highly homologous *B. amyloliquefaciens* enzyme with an epoxyalkyl glycoside inhibitor (*27*), and the X-ray structure of a covalent enzyme−inhibitor complex of a hybrid *B. amyloliquefaciens*−*B. macerans* enzyme with the same epoxyalkyl inhibitor (*28*). On these bases, the assignment of the residue acting as a general acid−base catalyst to E138 (*B. licheniformis*) was solely based on the fact that, in addition to E134, it was the only carboxylic amino acid residue conserved among the *Bacillus* 1,3-1,4-β-glucanases whose mutation to glutamine or asparagine yielded inactive enzymes (*26*). Inspection of the X-ray structure of the free enzyme (*19*) indicates that E138 is indeed suitably disposed for catalysis at 0.53 nm (O to O) from E134, but the absence of an X-ray structure of a reversible enzyme−inhibitor complex precludes the unambiguous assignment.

Despite the common claim of specific labeling of the nucleophile by epoxyalkyl glycosides (*27*), recent results on a retaining xylanase have shown that either the nucleophile or the general acid−base residues can be chemically derivatized by this type of affinity labels. Rouvinen and coworkers (*29*) reported, for the endo-1,4-xylanase II from *Trichoderma reesei*, that the alkyl chains of these ligands are quite flexible and are capable of adopting a range of different conformations. In fact, the reaction of an epoxyalkyl glycoside in the active site of a retaining glycosidase is initiated by proton donation to the epoxide oxygen, followed by the nucleophilic attack from another active site residue leading to the formation of a covalent bond between the ligand and enzyme. After donating the proton, the general acid residue also becomes a carboxylate anion, a putative nucleophile competing with the true nucleophile in the glycosidase mechanism. Then, depending on the length and con-

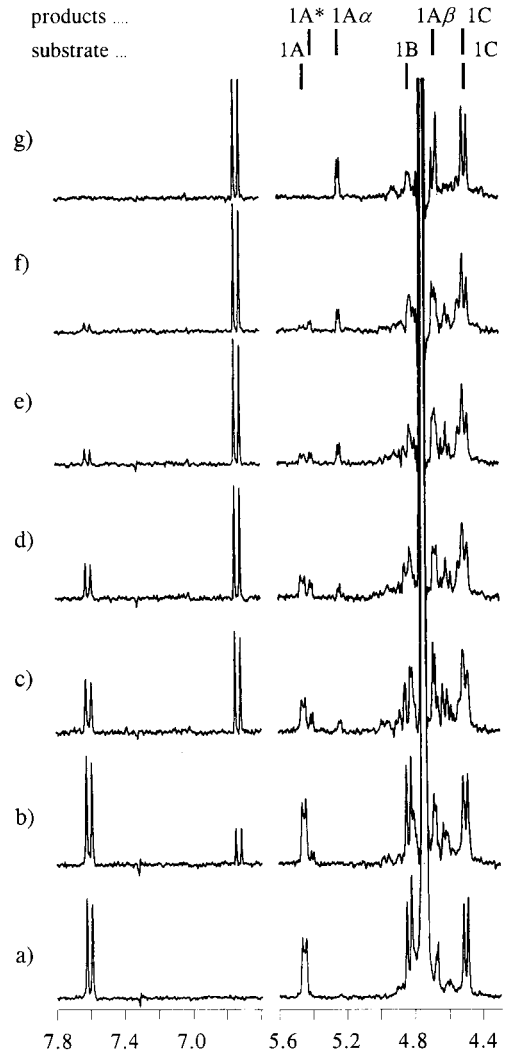


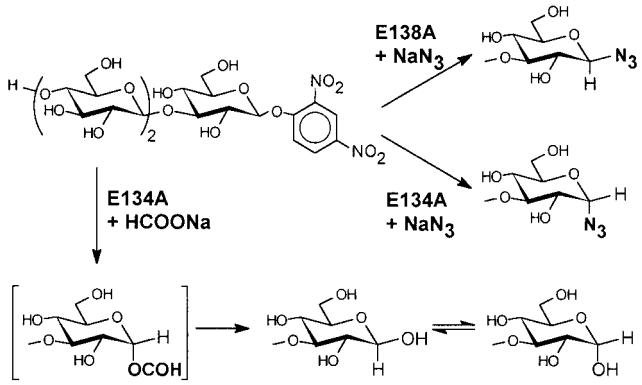
FIGURE 7: ^{1}H NMR monitoring of the reaction of G4G3G-2,4DNP with the 1,3-1,4-β-glucanase E134A mutant in the presence of sodium formate. The reaction was carried out in D_2O at 25 °C and pD 7.3, where [E] = 11.4 μM, [S] = 10 mM, and [formate] = 2 M: (a) G4G3G-2,4DNP substrate at $t = 0$ and (b−g) After 15 min and 2, 3, 5, 7, and 22 h. δ values are given in parts per million (J in hertz): 4.48 (8.1) H-1C substrate, 4.49 (8.0) H-1C hydrolysis product, 4.66 (8.1) H-1A hydrolysis product (β-anomer), 4.76 (8.1) H-1B hydrolysis product, 4.81 (8.1) H-1B substrate, 5.23 (3.5) H-1A hydrolysis product (α-anomer), 5.40 (7.5) H-1A α-glycosyl formate (asterisk in the spectra), 5.44 (7.5) H-1A substrate. Assignment of the aromatic signals (6.6−7.8 ppm) is given in Figure 5.

formation of the alkyl side chain, the epoxide compounds can react with both the nucleophile and the general acid−base catalyst of the glycosidase.

Therefore, the assignment of E134 as the catalytic nucleophile in *Bacillus* 1,3-1,4-β-glucanases based solely on the epoxide labeling experiments is not conclusive provided there is no functional proof of E138 being the general acid−base residue. In principle, E134 and E138 in the *B. licheniformis* enzyme (or the homologous glutamate residues in other *Bacillus* 1,3-1,4-β-glucanases) are the catalytic residues as proposed by site-directed mutagenesis studies and X-ray crystallography, but which one is the general acid−base and which one the nucleophile?

Chemical Rescue of Inactive Mutants. Here we extend the kinetic analysis approach first proposed by Withers and co-workers for exoglycanases [*Agrobacterium* β-glucosidase (*11*) or exoglucanase/xylanase from *Cellulomonas fimi* (*10*, *12*)] to a general chemical rescue methodology that can also apply to endoenzymes for providing functional evidence of the role of the essential catalytic residues. The rationale of the approach is summarized in Scheme 3.

Scheme 1: Structure of the Products from Chemical Rescue of the Reaction of G4G3G-2,4DNP with E138A and E134A by Azide and Formate

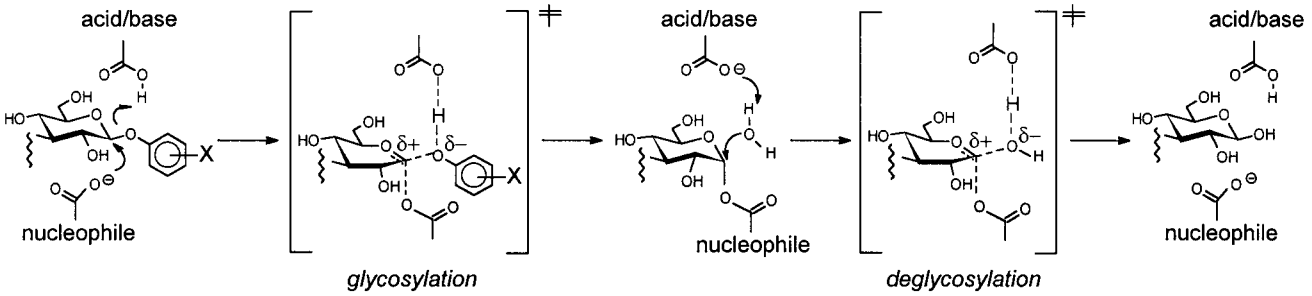


For a retaining β-glycosidase, removal of the general acid−base catalyst (Glu to Ala mutation) will have little or no effect on the first glycosylation step for an activated substrate that requires little or no general acid assistance, but the reaction will stop at the deglycosylation step. The glycosyl−enzyme intermediate will be slowly hydrolyzed in the absence of the general base to assist the attack of a water molecule. Addition of an exogenous nucleophile (such as azide) that does not require general base assistance may lead to an attack on the glycosyl−enzyme intermediate with the overall effect of enzyme reactivation. Since the exogenous nucleophile will operate on the deglycosylation step, the reaction will yield the β-glycosyl product.

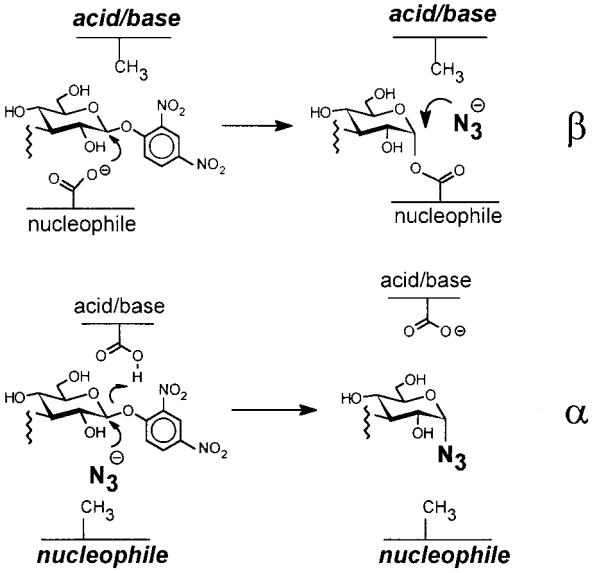
On the other hand, mutation of the nucleophile should render an inactive mutant where the first glycosylation step has been drastically slowed because of the inability of the mutant enzyme to form the glycosyl−enzyme intermediate. In this case, a cavity has been created in the active site on the α-face (Glu to Ala mutation) which can accommodate a small exogenous nucleophile such as azide. The enzyme might be reactivated through a different mechanism, following a single inverting displacement, to give the α-glycosyl product. Therefore, if reactivation occurs, the stereochemistry of the new glycosyl azide product formed upon chemical rescue of both alanine mutants will indicate which residue acts as the general acid−base and which operates as the nucleophile in the catalytic mechanism of the wild type enzyme.

Substrate Reactivity. Aryl β-glycosides of 3-*O*-β-cellobiosyl-D-glucopyranose have proven to be good substrates for the 1,3-1,4-β-glucanase from *B. licheniformis* (*22*). To select the appropriate substrates for the chemical rescue experiments, we had previously performed reactivity studies (Hammett analysis) on the wild type enzyme using a set of substituted aryl β-glycosides with different phenol-leaving group abilities as measured by the pK_a of the free phenol released upon enzymatic hydrolysis (*23*). Results are reproduced in Figure 8. The steady-state kinetic parameters show no simple dependence of reactivity on aglycon acidity. The Hammett plot (log k_{cat} vs pK_a of the aglycon) is biphasic

Scheme 2: Retaining Glycosidase Mechanism, Showing the Transition States of the Glycosylation and Deglycosylation Steps



Scheme 3: Chemical Rescue Methodology



with an upward curvature at low pK_a values. The mechanistic interpretation of this unusual behavior not seen before for other retaining glycosidases remains obscure, but suggests a change in transition-state structure depending on the aglycon (*23*). On the other hand, and more important in the context of this work, the negative slope in the k_{cat} versus pK_a plot for aryl β-glycosides with a pK_a for the leaving group of <7 indicates that the glycosylation step is at least partially rate-determining even for highly activated substrates; for deglycosylation to become rate-limiting, k_{cat} would level off for activated substrates since this step is independent of the aglycon.

On these bases, two substrates were chosen to perform the chemical rescue experiments: the 4-methylumbelliferyl glycoside (G4G3G-MU) as a representative of a poor substrate, for which glycosylation is rate-limiting, and the 2,4-dinitrophenyl glycoside (G4G3G-2,4DNP), which has a better leaving group and a 1000-fold higher k_{cat}, for which the glycosylation step might not be fully rate-limiting, and which would require little general acid assistance.

Effect of Azide as an Exogenous Nucleophile. Azide has no activation effect on the wild type enzyme; rather, it behaves as an inhibitor of the hydrolysis of both substrates. Addition of azide reactivates both mutants (E138A and E134A) in a concentration-dependent manner using the activated 2,4-dinitrophenyl glycoside but has essentially no effect on the hydrolysis of the poor G4G3G-MU substrate. Reaction monitoring by ^{1}H NMR spectroscopy shows unambiguously that E138A yields the β-glycosyl azide product, whereas E134A gives the α-glycosyl azide (Scheme 1). Therefore, on the basis of the rationale of the chemical rescue methodology, the results provide functional evidence of E138 being the general acid−base catalyst and confirm the proposal of E134 as the catalytic nucleophile.

The kinetic interpretation of the chemical rescue is more complex than in the few reported examples on exoglycosidases (*10*−*12*, *44*, *45*), the effect of azide on the kinetic parameters being vastly different compared to these examples.

E138A. Deletion of the carboxyl side chain of E138 is expected to slow both steps in the retaining glycosidase mechanism; it functions as an acid catalyst in the first step (glycosylation) and as a base in the second (deglycosylation). In this system, k_{cat}/K_M represents the first irreversible step, which is glycosylation, according to the scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_2} E{-}P' \xrightarrow{k_3} E + P$$

$$k_{cat} = k_2{\cdot}k_3/(k_2+k_3) \qquad k_{cat}/K_M = k_1{\cdot}k_2/(k_2+k_{-1})$$

$$K_M = k_3{\cdot}(k_2+k_{-1})/k_1{\cdot}(k_3+k_2)$$

where E·S is the noncovalent Michaelis complex, E−P′ is the covalent glycosyl−enzyme intermediate, and k_2 and k_3 are the glycosylation and deglycosylation rate constants, respectively.

Mutation of the general acid residue (WT to E138A) produces a 2000-fold reduction in k_{cat}/K_M with the activated substrate, and this parameter for the mutant is still 1000-fold higher than that with the poor substrate G4G3G-MU (Table 1). Therefore, glycosylation is also partially rate-limiting in the E138A mutant even for the activated substrate. Moreover, K_M values do not change significantly compared to those of the wild type, whereas a large drop would be expected if deglycosylation becomes rate-limiting (*10*). But the fact that E138A is rescued by azide when using G4G3G-2,4DNP but not with G4G3G-MU suggests that departure of the 2,4-dinitrophenyl leaving group requires little general acid assistance. Azide then reactivates the enzyme acting as a nucleophile in the second step (since it gives the β-glycosyl azide product), but it may also have an effect on the glycosylation step. Increasing concentrations of sodium azide result in progressive increases in k_{cat} and k_{cat}/K_M values. The increase in k_{cat} is easily interpreted as the result of increasing the deglycosylation rate constant k_3 with azide. However, the increase of k_{cat}/K_M is surprising. If the effect of azide were primarely on the deglycosylation step, and not on glycosylation, values of k_{cat}/K_M would remain essentially constant across the range of azide concentrations, since $k_{cat}/$

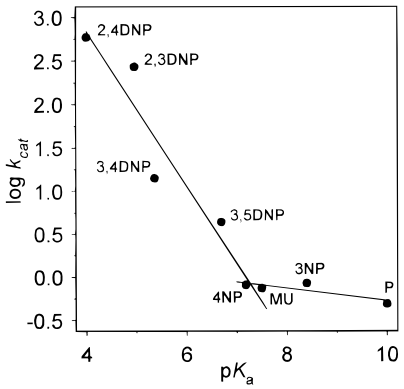


FIGURE 8: Hammett plot for the enzyme-catalyzed hydrolysis of substituted aryl β-glycosides by wild type 1,3-1,4-β-glucanase from *B. licheniformis*. Conditions were as follows: citrate/phosphate buffer and 0.1 mM $CaCl_2$ at pH 7.2 and 30 °C. Substrates were 2,4-dinitrophenyl (2,4DNP), 2,3-dinitrophenyl (2,3DNP), 3,4-dinitrophenyl (3,4DNP), 3,5-dinitrophenyl (3,5DNP), 4-nitrophenyl (4NP), 4-methylumbelliferyl (MU), phenyl (P), and β-glycosides of 3-*O*-β-cellobiosyl-D-glucopyranose. Data were taken from ref *23*.

K_M contains rate constants up to the first irreversible step. This result then suggests that azide also has a marked effect on a step preceding deglycosylation, either on the glycosylation step (increasing k_2) or on substrate binding to the mutant (modifying k_1 and/or k_{-1}). The large decrease observed on K_M with azide concentration is also consistent with an effect on k_2; if binding to form the noncovalent E·S complex is not significantly affected, K_M values decrease progressively as the ratio k_3/k_2 drops, indicating that azide produces a larger increase on k_2 than on k_3. According to that, the effect of azide on k_{cat} can be reinterpreted as the result of increasing the rates of both glycosylation (k_2) and deglycosylation (k_3). Another interesting observation is that k_{cat}/K_M for the mutant at high (saturating) azide concentrations approaches the value of the wild type enzyme with the same substrate. It suggests that the glycosylation rate constant k_2 has been increased to the wild type value. Activation on k_2 may be due to Brønsted acid catalysis by enzyme-bound HN_3. Hydrazoic acid may bind at the site of the excised carboxylic side chain, and its pK_a [4.7 in water (*46*)] may be higher in the hydrophobic active site environment than free in solution [the kinetic pK_a of Glu138 in the wild type enzyme is increased to 7.0 (*22*)]. As shown in Table 3, the activity of E138A in the presence of 1 M sodium azide increases with decreasing pH, where a larger fraction of azide exists in the protonated form, consistent with Brønsted acid catalysis by HN_3. If formation of the glycosyl−enzyme intermediate were fully rate-determining ($k_{cat} = k_2$), this pH profile would reflect the pK_a of enzyme-bound hydrazoic acid.

The kinetic results discussed above for the chemical rescue of the 1,3-1,4-β-glucanase mutant E138A are notably different from those reported for Ala or Gly mutants at the general acid−base residue in exoglycosidases [i.e., *C. fimi* exoglucanase/xylanase (*10*), *Agrobacterium faecalis* β-glucosidase (*11*), and *E. coli* β-galactosidase (*44*, *45*)]. In these systems, the deglycosylation step is rate-limiting for activated aryl glycoside substrates. For excellent substrates such as 2,4-dinitrophenyl glycosides, k_{cat}/K_M values are not significantly slowed by mutation, whereas for substrates requiring some general acid catalysis, there is a significant rate reduction. When azide was added as a nucleophile and 2,4-dinitrophenyl glycosides were used as substrates, both k_{cat} and K_M increased as a function of azide concentration. In contrast, k_{cat}/K_M remained virtually constant. These results were interpreted as a firm evidence that the effect of azide was on the deglycosylation step: (a) glycosylation is not significantly compromised for the mutant, and (b) the parameter k_{cat}/K_M reflects the first irreversible step, formation of the glycosyl−enzyme intermediate, and this step is not affected by an exogenous nucleophile.

In our case, the situation is quite different, allowing us to conclude that (a) the glycosylation step is partially rate-limiting for the mutant even with an activated substrate and (b) azide participates in both steps: it activates the deglycosylation step by acting as a nucleophile that does not require general base assistance to lead to the β-glycosyl azide product, and it also has a large effect on a step prior to the nucleophilic attack on the glycosyl−enzyme intermediate, probably increasing the rate of glycosylation through Brønsted acid catalysis by enzyme-bound HN_3.

E134A. Removal of the side chain acting as the catalytic nucleophile inactivates the enzyme in the first glycosylation step. The effect on the kinetic parameters is much larger than that observed for the E138A mutant. For the G4G3G-2,4DNP substrate, k_{cat} and k_{cat}/K_M are reduced 10^6-fold relative to those of the wild type enzyme. Azide reactivates the mutant through a single inverting displacement to give the α-glycosyl azide product. Both k_{cat} and K_M increase as a function of azide concentration. However, they do not follow a parallel variation, K_M showing a rapid increase at low azide concentrations and k_{cat} not reaching saturation up to 3.3 M azide. For a one-step inverting reaction, it may indicate that azide also affects substrate binding by increasing K_d.

Effect of Formate as an Exogenous Nucleophile. Carboxylic acids resemble more closely the structure of the removed glutamate side chains. Acetate and propionate had no effect on enzyme activity on both mutants, presumably because they cannot fit into the space created by removal of the carboxyl side chain. Formate, however, had a different behavior. The alanine mutant at the position of the general acid−base residue (E138A) was not rescued by formate, whereas the mutant at the nucleophile (E134A) showed a concentration-dependent activation.

In principle, formate would be able to compete with water in the deglycosylation step in the E138A mutant with an activated substrate (as in the case of azide) or act as a general base to catalyze the attack of a water molecule, besides the effect it might have on the glycosylation step. In the first case, the β-glycosyl formate aduct would be formed as the primary product; in the second, the normal hydrolysis reaction would be activated. None of them are observed, thus suggesting that the negatively charged formate ion is not sufficiently nucleophilic to compete with water and reactivate the enzyme as azide does, or that it is not able to bind properly (or it has a very low binding affinity) to act as a general base in the deglycosylation step. Glu138 in the wild type enzyme is located in a more hydrophobic environment than the residue acting as a nucleophile, as expected due to its higher pK_a value (*22*), and also observed by inspection of the X-ray structure of the free enzyme (*19*). A different behavior has been seen in other systems, as in

Agrobacterium β-glucosidase (*11*) or *E. coli* β-galactosidase (*45*), wherein addition of carboxylic nucleophiles such as formate and acetate restored activity to mutants lacking the general acid−base residue.

On the other hand, formate reactivates the reaction of the E134A mutant with G4G3G-2,4DNP. At the highest formate concentration assayed, k_{cat} is increased 3000-fold relative to that of the formate-free reaction, and it is only 300-fold lower than the k_{cat} value for the wild type enzyme with the same substrate. Since K_M does not change too much (≤2-fold), the same large reactivation is seen on k_{cat}/K_M. The role of formate in the reaction mechanism is proved by analysis of the products upon activation. While the outcome of the overall reaction gives the same hydrolysis product as the wild type reaction, ^{1}H NMR monitoring has shown the formation of a transient intermediate in sufficient concentration to be detected by NMR. On the basis of the chemical shift of the anomeric proton and the coupling constant, it is tentatively assigned to the α-glycosyl formate (Scheme 1). Accordingly, formate is able to bind into the cavity created by removal of the glutamate side chain and to adopt a similar conformation, thus playing the role of the removed enzyme nucleophile Glu134. This is a remarkable result representing the first case where a nonmodified sugar gives a long-lived covalent intermediate that mimics the proposed glycosyl−enzyme intermediate of retaining glycosidases.

Since the rates of α-glycosyl formate formation and hydrolysis are not faster than mutarotation of the reducing sugar under the experimental conditions used, both the β- and α-anomers of the hydrolysis product are detected simultaneously. It precludes the assignment of the primary product from hydrolysis of the transient intermediate. The β-anomer would be expected to form first, the α-anomer arising from mutarotation, if the α-glycosyl formate is normally hydrolyzed by the enzyme through general base catalysis by the carboxylate of Glu138 (equivalent to the normal deglycosylation step). Work to unambiguously characterize the transient intermediate and the true nature, chemical or enzymatic, of its hydrolysis is in progress.

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