

Mutated Barley (1,3)- β -D-Glucan Endohydrolases Synthesize Crystalline (1,3)- β -D-Glucans*

Received for publication, April 24, 2002, and in revised form, May 22, 2002
Published, JBC Papers in Press, May 22, 2002, DOI 10.1074/jbc.M203971200

Maria Hrmova[‡], Tomoya Imai^{§¶}, Simon J. Rutten^{‡||}, Jon K. Fairweather^{§**}, Ludovic Pelosi[§], Vincent Bulone[§], Hugues Driguez[§], and Geoffrey B. Fincher^{‡ ‡‡}

From the [‡]Department of Plant Science, University of Adelaide, Waite Campus, Glen Osmond, South Australia 5064, Australia, and the [§]Centre de Recherches sur les Macromolécules Végétales, UPR 38041, Grenoble Cedex 9 France

Barley (1,3)- β -D-glucan endohydrolases (EC 3.2.1.39), inactivated by site-directed mutagenesis of their catalytic nucleophiles, show autocondensation glucosynthetic activity with α -laminaribiosyl fluoride and heterocondensation glycosynthetic activity with α -laminaribiosyl fluoride and 4'-nitrophenyl β -D-glucopyranoside. The native enzyme is a retaining endohydrolase of the family 17 group and catalyzes glycosyl transfer reactions at high substrate concentrations. Catalytic efficiencies ($k_{\text{cat}} K_m^{-1}$) of mutants E231G, E231S, and E231A as glycosynthases are 28.9, 0.9, and $0.5 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$, respectively. Glycosynthase reactions appear to be processive and proceed with pH optima of 6–8 and yields of up to 75%. Insoluble products formed during the glycosynthase reaction appear as lamellar, hexagonal crystals when observed by electron microscopy. Methylation, NMR, and matrix-assisted laser desorption ionization time-of-flight analyses show that the reaction products are linear (1,3)- β -D-glucans with a degree of polymerization of 30–34, whereas electron and x-ray diffraction patterns indicate that these (1,3)- β -D-glucan chains adopt a parallel, triple helical conformation. The (1,3)- β -D-glucan triple helices are orientated perpendicularly to the plane of the lamellar crystals. The barley (1,3)- β -D-glucan glycosynthases have considerable potential for tailored and high efficiency synthesis of (1,3)- β -D-linked oligo- and polysaccharides, some of which could have immunomodulating activity, or for the coupling of (1,3)- β -D-linked glucosyl residues onto other oligosaccharides or glycoproteins.

(1,3)- β -D-Glucans are emerging as potentially valuable therapeutic agents. They have anti-bacterial and anti-viral effects, they are believed to stimulate blood coagulation, and they are reported to accelerate the healing of wounds (1, 2). In addition, (1,3)- β -D-glucans have attracted attention as carcinostatic agents (3) and as immunomodulators in human immunodeficiency virus therapy (4).

* This work was supported by grants from the Australian Research Council and the Grains Research and Development Corporation of Australia (to G. B. F.) and from the Centre National de la Recherche Scientifique (to H. D. and V. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of a postdoctoral fellowship from the French Ministry of Research for postdoctoral fellowships.

|| Recipient of an Australian Postgraduate Award.

** Recipient of a postdoctoral fellowship from the Centre National de la Recherche Scientifique.

‡‡ To whom correspondence should be addressed. Tel.: 61-8-8303-7296; Fax: 61-8-8303-7109; E-mail: geoff.fincher@adelaide.edu.au.

Although (1,3)- β -D-glucans can be isolated in good yield from a number of microbial sources (5), it is becoming apparent that their therapeutic value is dependent on fine structural detail, including molecular mass, the degree of substitution or branching, and three-dimensional conformation (6). In many cases, the (1,3)- β -D-glucans that can be easily isolated from biological sources do not meet these criteria. Thus, realization of their potential value in therapeutic applications will rely on the development of efficient methods for the synthesis of (1,3)- β -D-glucans with quite specific physical and chemical properties.

Chemoenzymic procedures for the synthesis of specific oligo- or polysaccharides are now available. In these procedures, the normal hydrolytic direction of glycoside hydrolases can be reversed, either through manipulation of reaction conditions or through targeted modification of the enzyme, so that the hydrolases actually form glycosidic linkages between donor and acceptor glycosides (7). Specificity is usually maintained in the biosynthetic direction. The application of retaining glycoside hydrolases for the synthesis of β -linked oligo- and polysaccharides encompasses two distinct approaches. The first is an "equilibrium" approach, which relies upon high substrate concentrations and/or lowered water concentrations to generate synthetic products. This approach offers very modest yields (about 10%) and suffers from a low degree of regioselectivity (8). The second, "kinetic" approach uses a relatively reactive glycosyl donor, typically an aryl glycoside or a glycosyl fluoride, and offers yields ranging from ~25% to about 60% (9, 10). The biosynthetic reactions can be performed either in an aqueous environment (11) or in hydro-organic media (12). The modest yields can be attributed to hydrolysis of the newly generated reaction products that are themselves substrates for the hydrolytic enzyme or to limited stability or denaturation of the enzyme in nonaqueous media.

These problems have largely been overcome through "glycosynthase" methodology, which is a variation of hydrolase-catalyzed chemoenzymic synthesis of glycosidic linkages first developed on the exo-acting *Alcaligenes faecalis* β -D-glucosidase/ β -D-galactosidase (13). Glycosynthases are generated by the replacement of the catalytic nucleophile of the glycoside hydrolase with a nonnucleophilic amino acid residue. This results in a hydrolytically inactive mutant and therefore completely eliminates the problem of hydrolytic cleavage of newly formed oligo- or polysaccharide products. However, the mutated enzymes can transfer a sugar moiety from an α -glycosyl fluoride donor molecule to substituted or unsubstituted glycoside acceptors. The reactive α -glycosyl fluoride donor mimics the glycosyl-enzyme intermediate observed for the wild-type glycoside hydrolase and may be generated prior to the reaction (13) or prepared *in situ* (14, 15). The glycosynthase reactions proceed

in aqueous buffers and offer much higher product yields (between 80 and 100%) than other chemoenzymic methods (8, 9).

Here we describe mutated (1,3)- β -D-glucan glycosynthases that are capable of efficient chemoenzymic synthesis of a variety of (1,3)- β -linked oligo- and polysaccharides. The glycosynthases were generated by site-directed mutagenesis of a cDNA encoding a family 17, retaining (1,3)- β -D-glucan endohydrolase from barley (*Hordeum vulgare*) that is capable of catalyzing transglycosylation reactions at high substrate concentrations.¹ The catalytic nucleophile, Glu²³¹, of the barley (1,3)- β -D-glucan endohydrolase isoenzyme GII (16, 17) was replaced with various other amino acid residues, and the resultant glycosynthases were purified after expression of the mutated cDNAs in *Escherichia coli*. The catalytic efficiencies and other kinetic properties of the mutated enzymes have been compared, and the chemical and physical properties of the polymeric products of the glycosynthase reaction are defined.

EXPERIMENTAL PROCEDURES

Materials—Orcinol; laminarin, a (1,3)- β -D-linked glucan of degree of polymerization (DP)² ~33 from *Laminaria digitata*; bovine serum albumin (fraction V); and 4'-nitrophenyl β -D-glucopyranoside (4NPGlc) were purchased from Sigma, and tryptone and yeast extract were from Becton Dickinson Co. (Sparks, MD). Microcon microconcentrators were from Amicon (Beverly, MA), Kieselgel 60 thin layer plates were from Merck, and (1,3)- β -D-oligosaccharides of DP 2–7 were from Seikagaku Kogyo Co. (Tokyo, Japan). The pET 3a-HT expression vector and *E. coli* strain BL21 (DE3) pLysS were from Novagen (Madison, WI), *E. coli* strain XL-1 Blue used for construction of mutated cDNAs was from Stratagene (La Jolla, Ca, USA), and the pET 3a-HT/(1,3)- β -D-glucanase isoenzyme GII cDNA plasmid was constructed by Dr. Richard Stewart (18). Paramylon was a generous gift from Professor Bruce Stone (La Trobe University, Bundoora, Victoria, Australia).

Purification of Wild Type Barley (1,3)- β -D-Glucan Endohydrolase—Barley (1,3)- β -D-glucan endohydrolase isoenzyme GII was purified from a homogenate of 2-day-old seedlings by fractional precipitation with ammonium sulfate, ion exchange chromatography, chromatofocusing, and size exclusion chromatography as described previously (19). The purity of the (1,3)- β -D-glucan endohydrolase was assessed by SDS-PAGE, where a single protein band was detected at high protein loadings. The purity was confirmed by NH₂-terminal amino acid sequence analysis. No secondary amino acid sequences were detected in the first 52 amino acid residues, and quantification of recoveries during the sequence analysis showed that they were close to theoretical values (data not shown).

Generation of Mutated (1,3)- β -D-Glucanases E231A, E231G, and E231S—All mutants were generated using a procedure modified from Stratagene's QuikChange site-directed mutagenesis kit. The template DNA consisted of the wild type (1,3)- β -D-glucan endohydrolase isoenzyme GII cDNA inserted in the *NdeI/SacII* site of a pET 3a expression vector modified by Dr. Helen Healy (University of Adelaide, Australia) to include expression of a thrombin cleavage site followed by a His₆ purification tag at the COOH terminus. Approximately 0.5 μ g of template DNA was added to a PCR containing 190 ng of both forward and reverse orientation mutagenic primers and amplified with *PfuI* DNA polymerase. Forward and reverse primers, respectively, for the PCRs were as follows: 5'-G GTG GTG GGT TCG GCG AGC GGG TGG CCG TCG G-3' and 5'-C CGA CGG CCA CCC GCT CGC CGA CAC CAC CAC C-3' (for mutant E231A); 5'-G GTG GTG GTG TCG GCG AGC GGG TGG CCG TCG G-3' and 5'-C CGA CGG CCA CCC GCT CCC CGA CAC CAC C-3' (for mutant E231G); 5'-G GTG GTG GTG TCG TCG AGC GGG TGG CCG TCG G-3' and 5'-C CGA CGG CCA CCC GCT CGA CGA CAC CAC C-3' (for mutant E231S).

After PCR amplification (25 cycles of 95 °C for 1 min, 52 °C for 1.5 min, and 68 °C for 20 min), the reaction mixture was treated with *DpnI* restriction endonuclease to digest methylated template DNA. Remaining PCR-synthesized mutated plasmid was transformed into competent *E. coli* XL-1 Blue cells via a 45-s heat shock at 42 °C. Dideoxyribonucle-

otide sequencing (20) was used to confirm the sequence changes in mutated cDNAs. Wild type and mutated cDNAs were expressed in the pET 3a vector in *E. coli* strain BL21 (DE3) pLysS induced with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside (18). Cells were lysed by freeze-thawing, followed by sonication in 50 mM sodium phosphate buffer, pH 7.8, containing 300 mM NaCl and 20 mM imidazole. Insoluble proteins and cell debris were removed by centrifugation (16,000 \times g, 30 min), and soluble proteins were fractionated through a pre-equilibrated nickel-nitritotriacetic acid affinity column from Qiagen (Valencia, CA). The column was washed three times in 50 mM sodium phosphate buffer, pH 7.8, containing 300 mM NaCl and 20 mM imidazole, before elution of bound proteins with 270 mM imidazole in the same buffer. Purities of wild type and mutated enzymes were assessed by SDS-PAGE.

Protein Determination, SDS-PAGE, and Amino Acid Sequencing—Protein determination, SDS-PAGE, and amino acid sequence analysis were performed as described previously (21).

Polymerization of β -Laminaribiosyl Fluoride by Wild Type (1,3)- β -D-Glucan Endohydrolase—Freshly deacetylated β -laminaribiosyl fluoride (28.1 μ mol, 10 mg) in NaOMe/MeOH (12) was dissolved in a mixture of 50 mM sodium acetate buffer, pH 5.0 (0.2 ml) and 0.3 ml of MeCN and incubated under gentle shaking in the presence of a wild type (1,3)- β -D-glucan endohydrolase (3.1 pmol, 0.1 μ g) at 37 °C for 4 h. A further aliquot of the enzyme was added after 4 h. The mixture was separated on Kieselgel 60 thin layer plates, developed in ethyl acetate/acetic acid/water (2:1:1; by volume), and the reducing sugar products were detected with the orcinol reagent (19). Products of polymerization reactions with β -laminaribiosyl fluoride as a donor molecule and laminaribiose or 3-thiolaminaribiose as acceptor molecules were also investigated at donor/acceptor ratios of 1 or 10. The conditions of heterocondensation reactions were identical to those described above.

Polymerization of α -Laminaribiosyl Fluoride by Mutated (1,3)- β -D-Glucan Endohydrolases—(1,3)- β -D-Glucan endohydrolase mutant E231G (6.2 nmol, 0.2 mg) was added to a solution of a freshly deacetylated (22) α -laminaribiosyl fluoride (3; see Scheme 1) (175.8 μ mol, 60 mg) in 0.25 M phosphate buffer, pH 7.0 (3 ml), and the mixture was incubated at 37 °C for 12 h. The white precipitate that formed was recovered by centrifugation, and the pellet was thoroughly washed with water and lyophilized to yield (3)- β -D-Glc-(1,)_n (7) as a white powder (45 mg, 75% yield). This residue was dissolved in Me₂SO/D₂O and lyophilized (three times) prior to NMR analysis.

Preparation of 3-Thio- α -Laminaribiosyl Fluoride 6—A solution of octa-O-acetyl-3-thio- β -laminaribiose (4) (0.2 mmol), prepared as described (23), was dissolved in hydrogen fluoride/pyridine (3 ml of 7:3 (v/v)) and stirred at 0 °C for 4 h (Scheme 1). The solution was diluted with CH₂Cl₂ and poured onto ice-cold aqueous NH₃ (3 M), and stirring was continued for 30 min. The organic phase was diluted with CH₂Cl₂, evaporated, and purified by flash chromatography (40–50% EtOAc/petrol), to yield hepta-O-acetyl-3-thio- α -laminaribiosyl fluoride (5) as fine needles (98 mg, 82% yield), m.p. 163–164 °C (EtOH). ¹H NMR (CDCl₃, 300 MHz) δ 5.69 (dd, 1 H, $J_{1,2} = 2.4$ Hz, $J_{1,F} = 53.5$ Hz, H-1^H), 5.16 (t, 1 H, $J_{2,3} = J_{3,4} = 9.1$ Hz, H-3^H), 5.06–4.85 (m, 4 H, H-2^H, H-2^H, H-4^H, H-4^H), 4.73 (d, 1 H, $J_{1,2} = 10.2$ Hz, H-1^H), 4.26–4.18, 4.12–4.06 (2m, 5 H, H-5^H, H-6^H, H-6^H), 3.69 (ddd, 1 H, $J_{4,5} = 10.0$ Hz, $J_{5,6a} = 4.9$ Hz, $J_{5,6b} = 2.4$ Hz, H-5^H), 3.20 (t, 1 H, $J_{2,3} = J_{3,4} = 10.8$ Hz, H-3^H), 2.19–1.99 (7s, 21 H, 7 \times Me). ¹³C NMR (CDCl₃) δ 170.47–169.00 (7s, 7 \times CO), 103.30 (d, $J_{1,F} = 230$ Hz, C-1^H), 83.18, (C-3^H), 75.71, 73.70, 70.32, 68.04, 65.22 (C-2^H, C-3^H, C-4^H, C-4^H, C-5^H), 71.7 (d, $J_{2,F} = 25$ Hz, C-2^H), 71.0 (d, $J_{5,F} = 4.6$ Hz, C-5^H), 61.82, 61.68 (C-6^H, C-6^H), 20.65–20.44 (7s, 7 \times Me). HRMS: calculated for C₂₆H₃₅FO₁₆S (M + Na)⁺ 677.1528, found 677.1536. A suspension of the heptaacetate 5 (212 mg) in MeOH (10 ml) was treated with NaOMe (100 μ l, 0.1 M), and the mixture was stirred at progressively higher temperatures from 0 to 25 °C until dissolution was complete. The solution was neutralized with Amberlite IR-120 resin (H⁺ form); the resin was removed by filtration, and the filtrate was concentrated. The residual oil was lyophilized from H₂O to give 3-thio- α -laminaribiosyl fluoride (6) as colorless foam (112 mg). MS: m/z 383 (M + Na)⁺.

Polymerization of 3-Thio- α -laminaribiosyl Fluoride by Mutated (1,3)- β -D-Glucan Endohydrolase—(1,3)- β -D-Glucan endohydrolase E231G (3.1 nmol, 0.1 mg) was added to a solution of 3-thio- α -laminaribiosyl fluoride (6) (175.8 μ mol, 60 mg) in 0.25 M sodium phosphate buffer, pH 7.0 (1.4 ml), and the combined mixture was incubated (37 °C, 24 h) with mild shaking (Scheme 1). After 24 h, a further aliquot of enzyme (3.1 nmol, 0.1 mg) was added, and incubation continued for 24 h before MeOH (2.8 ml) was added to the mixture. The precipitate was collected by centrifugation, thoroughly washed with H₂O and reprecipitated (MeOH; three times) before lyophilization to yield (3)- β -D-Glc-S-(1,3)- β -

¹ M. Hrmova and G. B. Fincher, unpublished data.

² The abbreviations used are: DP, degree of polymerization; 4NPGlc, 4'-nitrophenyl β -D-glucopyranoside; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TEM, transmission electron microscopy.

D-Glc-(1, n) (7) as an off-white colored powder (30 mg, 50%). This residue was further lyophilized (D_2O , three times) prior to NMR analysis.

Initial Rates of Glycosynthetic Reactions—Initial rates of (1,3)- β -D-glucan endohydrolase E231A, E231G, and E231S mutated enzymes were measured in the presence of 14.7 mM α -laminaribiosyl fluoride or 3-thio- α -laminaribiosyl fluoride (autocondensation reactions) or in the presence of 14.7 mM α -laminaribiosyl fluoride and a 5-fold excess (74 mM) of 4NPGlc (heterocondensation reaction). Freshly deacetylated α -laminaribiosyl fluoride was dissolved in 150 mM phosphate/citrate buffer, pH 6.2, containing 160 μ g/ml bovine serum albumin, with or without 4NPGlc, and incubated with 36–136 μ mol of the purified mutated enzymes at 30 °C with gentle shaking. An Orion fluoride electrode

(Orion Research, Beverly, MA) interfaced with a pH/ion meter was used to determine the fluoride release and thus to deduce the glycosynthetic reaction rates. All enzymic reactions were corrected for spontaneous hydrolysis of α -laminaribiosyl fluoride.

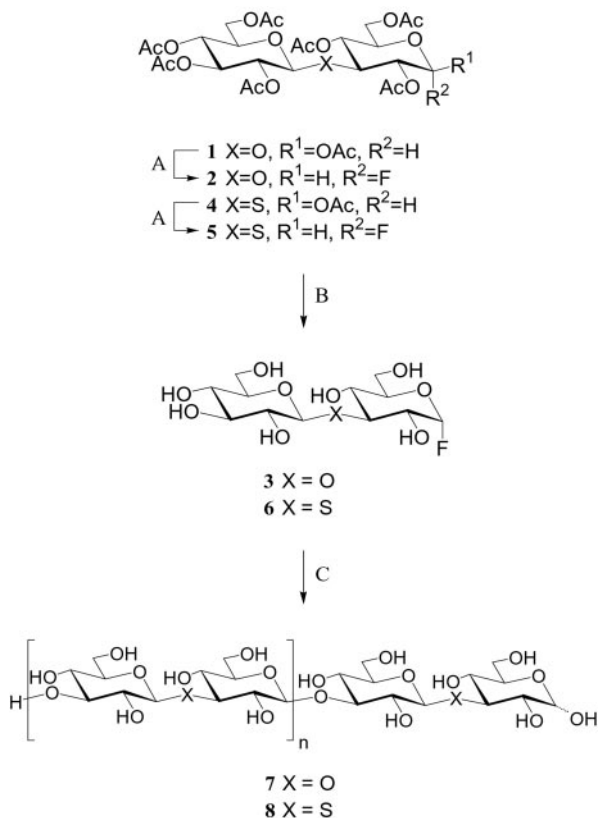
Kinetic Analyses of Glycosynthase Reactions—Kinetic parameters of (1,3)- β -D-glucan endohydrolase E231A, E231G, and E231S mutated enzymes were determined at 30 °C by incubating 18–108 pmol of the enzymes in 150 mM sodium phosphate/citrate buffer, pH 6.2, containing 160 μ g/ml bovine serum albumin. Initial rates of hydrolysis were determined in triplicate at substrate concentrations ranging from 0.1 to 3 times the K_m values. An Orion fluoride electrode interfaced with a pH/ion meter was used to determine the fluoride release. S.D. values, which were ~10%, were calculated according to Samuels (24). Kinetic data were processed by a proportional weighted fit using a nonlinear regression analysis program based on the Michaelis-Menten model equation (25). The initial enzyme concentration $[E]_0$ was $\ll [S]_0$, where $[E]_0$ and $[S]_0$ represent enzyme and substrate concentrations at zero time, and initial reaction rates were therefore measured in all cases.

pH Optima of Glycosynthase Reactions—The pH optima were determined over the range 3.0–9.3 in 150 mM sodium phosphate/citrate buffer containing 160 μ g/ml bovine serum albumin, with 18–80 μ mol of purified mutated enzymes at 30 °C under gentle shaking. The glycosynthetic rates were monitored with the fluoride electrode. The progress of the reactions was corrected for spontaneous hydrolysis of α -laminaribiosyl fluoride.

Restoration of Hydrolytic Activities of Glycosynthases by Anionic Nucleophiles—Reaction mixtures containing 0.2% (w/v) laminarin and 0–3.2 M sodium formate or sodium azide were incubated with 30–60 pmol of (1,3)- β -D-glucan endohydrolase E231G, E231A, and E231S mutated enzymes. The mixtures were incubated for up to 60 min at 30 °C with gentle shaking, in triplicate, in 200 mM sodium acetate buffer, pH 5.0, containing 160 μ g/ml bovine serum albumin. The rates of hydrolysis of laminarin were estimated from the increase in reducing sugars (19), after correction for background reducing levels of laminarin. One unit of hydrolytic activity is defined as 1 μ mol of glucose equivalents released per min. One unit corresponds to 16.67 nanokatal.

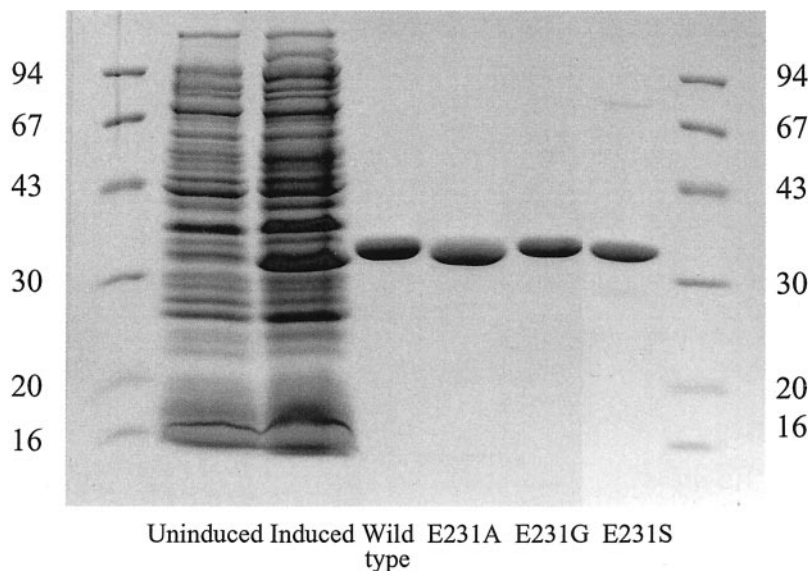
MALDI-TOF Ionization Mass Spectrometry—The molecular masses of the O- and S-linked polymeric products were determined on a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA). The molecular masses of the compounds were estimated from the m/z ratio of the quasimolecular-ionic $(M + Na)^+$ adducts.

Methylation Analysis—Products from the O-series glycosynthase reactions were methylated and analyzed for linkage type by the permethylated alditol acetate procedure (26). Partially methylated monosaccharides released by trifluoroacetic acid hydrolysis were reduced with NaBD₄ and acetylated. The resulting methylated alditol acetates were separated on a HP-6890 series gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a CP-Sil5 Chrompak capillary column (25 m \times 0.25 mm inner diameter; Supelco), using a temperature gradient (165–230 °C; 2.5 °C min⁻¹). The derivatives were identified by electron impact mass spectrometry using a NERMAG R-1010C spectrometer (Scientific Instruments, Ringoes, NJ), equipped with a HP5973 mass



SCHEME 1. Schematic showing the synthesis of the disaccharide fluoride donors, 3 and 6, and their polymerization. A, hydrogen fluoride/pyridine (80%, v/v); B, NaOMe/MeOH; C, mutated E231G (1,3)- β -D-glucanase in phosphate buffer (0.25 M, pH 7.0), 37 °C (7, 75%; 8, 50%).

FIG. 1. SDS-PAGE of purified wild type and mutated enzymes expressed in *E. coli*. Lanes 1 and 8, molecular mass markers; lane 2, crude *E. coli* uninduced extract; lane 3, crude *E. coli* induced extract; lane 4, wild type; lane 5, E231A; lane 6, E231G; lane 7, E231S. Approximately 10 μ g of protein was loaded in each case.



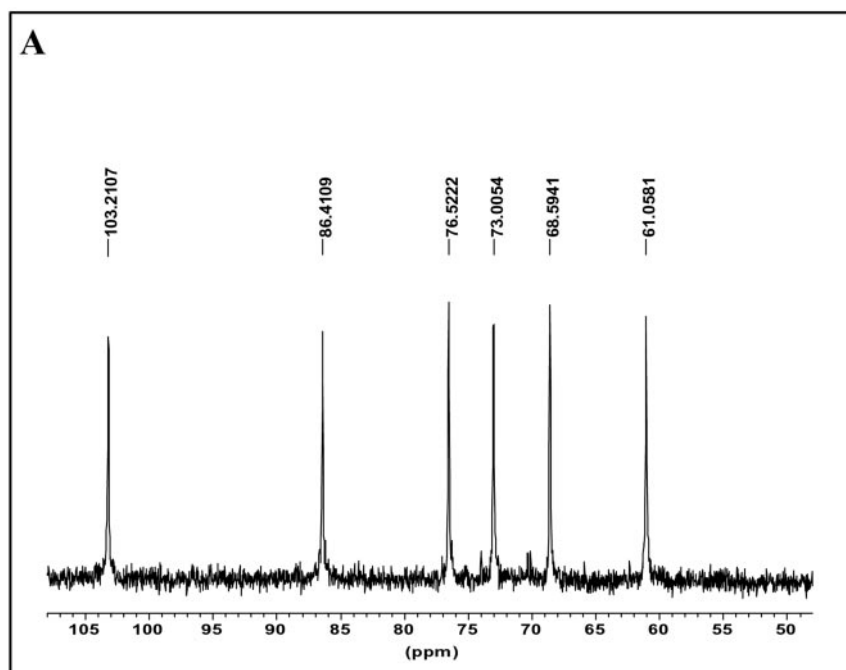
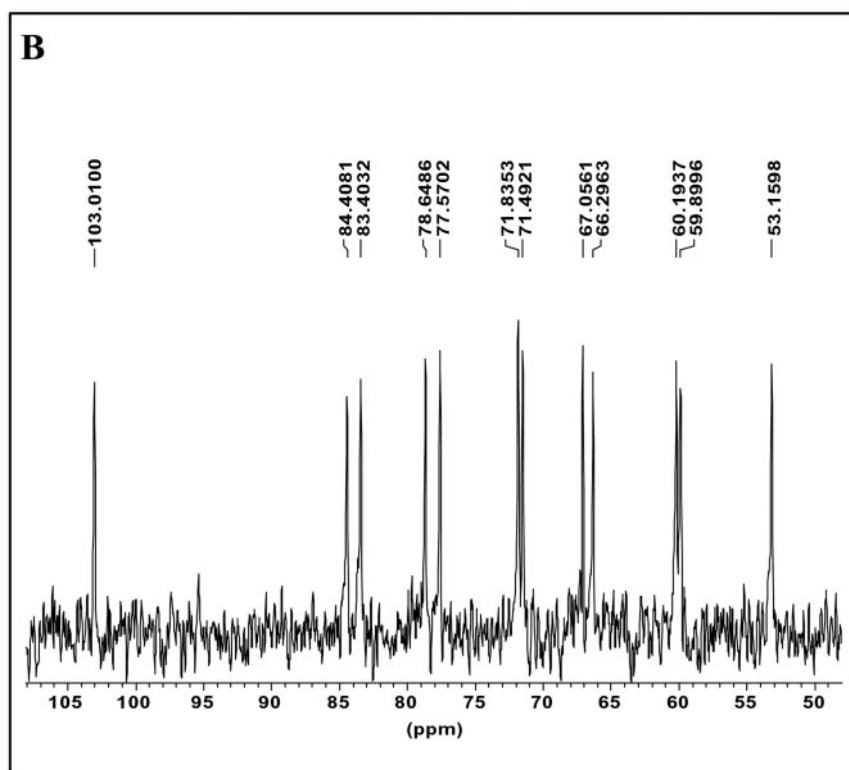


FIG. 2. ^{13}C NMR spectra of two (1,3)- β -D-linked polymers synthesized by mutated (1,3)- β -D-glucanase E231G. A, (3)- β -D-Glc-(1, n)_n (7); B, (3)- β -D-Glc-S-(1,3)- β -D-Glc-(1, n)_n (8).



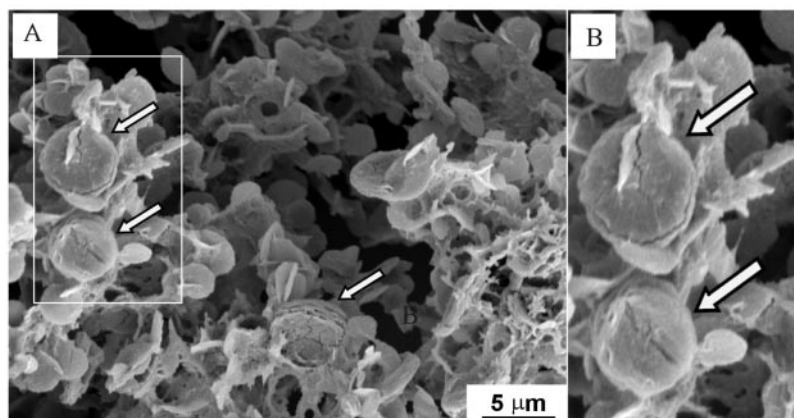
selective detector and using *myo*-inositol hexaacetate as an internal standard (27).

Electron Microscopy—The lyophilized product was fixed on a stub using conductive glue and coated with gold/palladium alloy by ion-sputtering prior to observation. Scanning electron microscopy examinations were made with a JEOL JSM-6100 microscope at an accelerating voltage of 10 kV and a working distance of 16 mm. For transmission electron microscopy, droplets of aqueous suspensions of the dialyzed product were deposited onto carbon-coated copper grids and negatively stained with 2% (w/v) uranyl acetate. Samples were observed with a Philips CM200 cryoelectron microscope at an accelerating voltage of 80

kV. Selected area electron diffraction was performed using the non-stained sample over an area 1 μm in diameter and at an accelerating voltage of 200 kV. The thickness of the crystals corresponding to the glycosynthase product was estimated using latex beads having a diameter of 0.109 μm as a reference, after shadowing the sample with a tungsten/tantalum alloy.

X-ray Diffraction Analysis—Lyophilized product was packed in a thin wall x-ray glass capillary (0.5-mm diameter) and mounted in a flat Wharfedale vacuum camera equipped with a 0.25-mm pin hole collimator. The sample was subjected to nickel-filtered Cu-K α radiations generated at 30 kV and 20 mA. The resulting x-ray diffraction diagrams were

FIG. 3. Scanning electron microscopy/TEM micrograph of the product of autocondensation reaction synthesized by mutated (1,3)- β -D-glucanase E231G from α -laminaribiosyl fluoride. A, arrows point to several stacking disks of (1,3)- β -D-glucan; B, close-up view of the designated area from A.



recorded at room temperature on Kodak DEF-5 films. For comparison, the x-ray diffraction pattern of paramylon, a highly crystalline (1,3)- β -D-glucan from *Euglena gracilis*, was obtained under similar conditions.

Molecular Modeling—The three-dimensional molecular model of (1,3)- β -D-glucanase mutant E231G was built from a wild type enzyme (Protein Data Bank accession code 1GHS; Ref. 17) using the software O (28), Modeler, version 6.1 (29), or Swiss-Pdb viewer (30). The models of the three mutated enzymes were further checked manually on O and GRASP (31) and were essentially identical. Models of molecular surfaces of wild type and mutated enzymes were generated with a probe radius of 1.4 Å. Electrostatic potentials were calculated using the Poisson-Boltzmann equation with GRASP.

RESULTS

“Transglycosylation” Activity of the Wild Type Enzyme—In hydro-organic media, the wild type (1,3)- β -D-glucan endohydrolase, purified from barley seedlings or expressed in a heterologous expression system of *E. coli*, converted β -laminaribiosyl fluoride into (1,3)- β -D-linked oligosaccharides in the presence or absence of acceptors (data not shown). However, the overall yields of the products with β -laminaribiosyl fluoride (about 20%) were consistently low with the wild type (1,3)- β -D-glucan endohydrolase (data not shown), in agreement with previously published data (12).

Generation and Isolation of Mutated Enzymes—Mutations causing amino acid substitutions E231G, E231S, and E231A were introduced at the cDNA level using site-directed mutagenesis and confirmed by DNA sequence analysis. The three mutated (1,3)- β -D-glucan endohydrolases were expressed in *E. coli* and purified by affinity chromatography on nickel-nitrilotriacetic acid resin. Single protein bands were observed following SDS-PAGE (Fig. 1). The expressed wild type and mutated enzymes had molecular masses of 33–34 kDa (Fig. 1), which corresponded to that calculated for the barley enzyme (32). Yields of the recombinant enzymes varied for each mutant and between experiments but were generally 1.5–6.6 mg of protein per liter of bacterial culture. The expressed wild type enzyme exhibited a specific activity of 220 units mg^{-1} protein, which is similar to values reported for (1,3)- β -D-glucan endohydrolase isoenzyme GII preparations purified from barley seedlings (19). In contrast, no activity against laminarin, a (1,3)- β -D-linked glucan of DP \sim 33 (5), could be detected for the three mutated enzymes, even after prolonged incubation with the substrate. When the expressed wild type and mutated enzymes were stored at 4 °C at a concentration of 0.15–1.2 mg/ml in 50 mM sodium phosphate buffer, pH 7.8, containing 300 mM NaCl and 270 mM imidazole, no appreciable loss of hydrolase or glycosynthase activity was observed over several weeks.

Glycosynthase Activity of the Mutated Enzymes—When the expressed, mutated (1,3)- β -D-glucan endohydrolases E231G, E231A, and E231S were incubated with α -laminaribiosyl fluoride, a white flocculant precipitate formed within 2–24 h. Pre-

cipitation was notably faster with the E231G mutant and slowest with the E231A mutant. Preliminary analyses of the supernatant from the E231G reaction by thin layer chromatography showed the depletion of the α -laminaribiosyl fluoride substrate and the accumulation of carbohydrate that remained at the origin (data not shown). After prolonged incubation, a yield of 75% was calculated, on the basis that 0.2 mg of the E231G mutant converted 60 mg of α -laminaribiosyl fluoride to 45 mg of insoluble product. Incubation of the (1,3)- β -D-glucan glycosynthase E231G with 3-thio- α -laminaribiosyl fluoride also resulted in the formation of insoluble material, albeit at a much slower rate.

^{13}C NMR Spectrometry of the Glycosynthase Products—The precipitate produced during the glycosynthase reaction with the E231G mutant was washed and collected by centrifugation and analyzed by ^{13}C NMR spectrometry. The spectrum of this product (Fig. 2A) was similar in all respects to the reported spectrum of curdlan (33), which is an essentially linear (1,3)- β -D-glucan. The glycosynthase product was therefore an essentially unsubstituted homopolymer of (1,3)- β -linked D-glucosyl residues.

When 3-thio- α -laminaribiosyl fluoride was incubated with E231G, no visible precipitate formed after 24 h, and thin layer chromatographic analysis of the mixture showed unreacted 3-thio- α -laminaribiosyl fluoride. The addition of more enzyme and incubation for a further 24 h resulted in the disappearance of most of the 3-thio- α -laminaribiosyl fluoride, although concurrent decomposition of the substrate was also evident (data not shown). This result indicated that the replacement of the conventional O-glycosidic linkage with an S-glycosidic linkage between the donor subsites –2 and –1 was less favorable for ligand recognition. Nonetheless, a precipitate could be collected from this mixture by the addition of methanol, and although this product was likely to be of lower DP than that produced with α -laminaribiosyl fluoride, ^{13}C NMR analysis (Fig. 2B) revealed a repeating disaccharide unit of 12 carbon atoms (3)- β -D-Glc-S-(1,3)- β -D-Glc-(1,) $_n$, in which every second (1,3)- β -D-glucosyl residue was connected through a sulfur atom (δ 103 ppm for C1-O; 84.4 ppm for C1-S; 83.4 ppm for O-C3, and 53.2 ppm for S-C3). In contrast with the rapid hydrolysis of the O-linked product following incubation with wild type (1,3)- β -D-glucan endohydrolase, the product from 3-thio- α -laminaribiosyl fluoride was hydrolyzed very slowly.

Degree of Polymerization of the Glycosynthase Product (Compound 7)—Methylation analysis of the glycosynthase product yielded one major permethylated alditol acetate (96.8%), which was identified by electron impact mass spectrometry as 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol (data not shown). This indicated that the reaction product was essentially a linear (1,3)- β -linked glucan. The ratio between the major deriva-

tive, which corresponded to (1,3)-D-glucosyl residues, and a minor derivative that corresponded to terminal nonreducing end glucosyl residues (3.1%, identified as 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol; data not shown) indicated that the (1,3)- β -D-glucan product of the glycosynthase reaction had an average DP of about 30.

MALDI-TOF mass data of compound **7** showed a bell-shaped curve, with a range of DP from 28 to 44. The major components had DPs of 30 and 34 (data not shown). For the product formed by mutant E231G with the 3-thio- α -laminaribiosyl fluoride, MALDI-TOF mass spectrometry showed a mixture of oligosaccharides with DPs ranging from 6 to 18 (data not shown). The lower average DP of the latter products was consistent with the absence of precipitated reaction products when the 3-thio- α -laminaribiosyl fluoride substrate was used.

Electron Microscopy—Scanning electron microscopy of the insoluble glycosynthase product formed when mutated enzyme E231G was incubated with α -laminaribiosyl fluoride revealed the presence of disks ranging from 1 to 5 μ m in diameter (Fig. 3A). While the thickness of a single disk was too small to be accurately determined, in some cases larger aggregates that probably resulted from stacking of many disks could be observed (Fig. 3B). Repeated observations indicated that most of the glycosynthase product was in the disk form.

Initial examination of the glycosynthase product by transmission electron microscopy (TEM) revealed dark masses that presumably corresponded to the aggregates observed by scanning electron microscopy. However, well defined hexagonally shaped platelets could be seen at the edges of the aggregates (Fig. 4) and are likely to represent the individual disks identified by scanning electron microscopy (Fig. 3). A very minor proportion of the glycosynthase product, located at the edge of some aggregates observed by TEM, exhibited a microfibrillar appearance (data not shown).

Electron Diffraction Pattern—The hexagon-shaped crystals resembled the lamellar crystallites previously observed in a sample of recrystallized curdlan with a degree of polymerization of 50 (34). Selected area electron diffraction patterns were therefore collected from the hexagon-shaped platelets. The hexagonal diffraction pattern so obtained (Fig. 4A, inset) corresponded to the h^*k^*0 reciprocal net of the crystal structure of anhydrous, triple helical (1,3)- β -D-glucan (35, 36). Thus, the electron diffraction pattern provided direct evidence that the (1,3)- β -D-glucan platelets formed in the glycosynthase reaction were lamellar single crystallites having a hexagonal unit cell, where the crystallite grows two-dimensionally along the $(hk0)$ plane (34). The size of single hexagonal crystallites (2–3 μ m in diameter; Fig. 4A) matched those of the disks observed in the scanning electron microscopy photographs (Fig. 3).

The electron diffraction pattern shows that the (1,3)- β -D-glucan triple helices are organized perpendicularly in the lamellar crystallite, as illustrated in Fig. 4B. The average thickness of the crystallites was estimated by TEM with shadowing to be 8 ± 3 nm (data not shown). The absence of further crystallite growth along the c axis suggests that the (1,3)- β -D-glucan product of the glycosynthase reaction is itself an average of 8 nm in length and perhaps has “ragged” ends that prevent growth in the c direction. Based on a pitch of 0.2835 nm/residue for (1,3)- β -D-glucan helices (35, 36), the degree of polymerization of the product that spans the 8-nm crystallites is estimated to be about 28, which is close to the values obtained by methylation and MALDI-TOF mass spectrometric analyses. This is consistent with an extended chain crystal model for the synthesized (1,3)- β -D-glucan rather than a folded chain crystal in which a single molecular chain in the lamellar crystallite folds back into the crystallite.

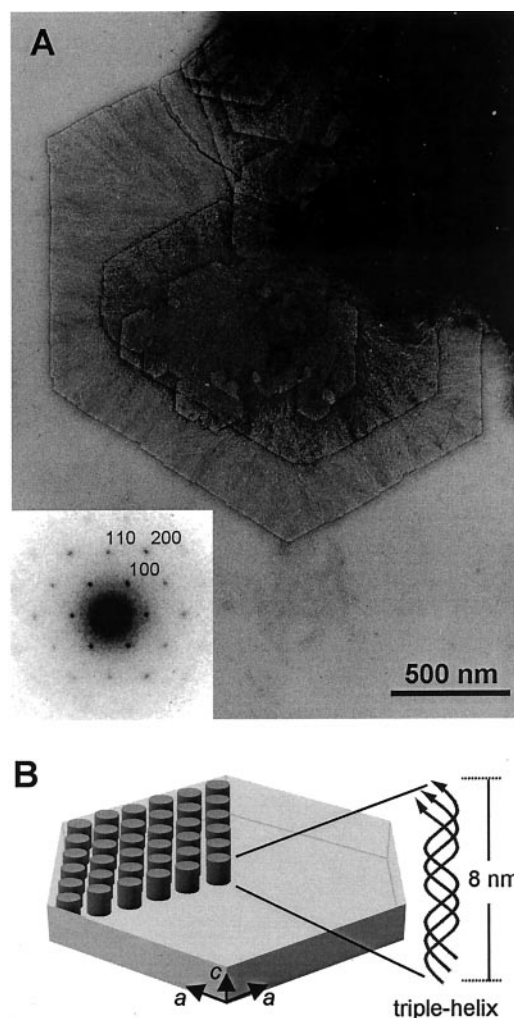


FIG. 4. Scanning electron microscopy/TEM micrograph of the products synthesized by mutated (1,3)- β -D-glucanase E231G during an autocondensation reaction. A, images taken after negative staining of *in vitro* product with uranyl acetate. The inset in A represents electron diffraction diagram of the crystallite with the hexagonally shaped lamellar structure in the 001 projection. B, schematic representation of the triple helices packed perpendicularly to the plane of a hexagonal lamellar crystallite. a and c represent unit cell axes. The arrows in the triple helix indicate the individual (1,3)- β -D-glucan chains are parallel.

X-ray Diffraction Pattern—The powder diagrams obtained by x-ray diffraction analysis of the highly crystalline, triple helical (1,3)- β -D-glucan, paramylon, and of the glycosynthase product are compared in Fig. 5. The pattern for the glycosynthase product (right sector) was of relatively poor resolution but nevertheless exhibited reflections seen for anhydrous paramylon (left sector). This confirmed the presence of a (1,3)- β -D-glucan with a triple helical crystalline structure (35, 36). The x-ray diffraction diagram of paramylon, which forms three-dimensional crystalline granules, is characterized by three intense reflections (100, 111, and 201). The innermost reflection (100) corresponds to the periodicity of the hexagonal arrangement of the triplex in the 001 projection and is also well defined in the glycosynthase product. The reflections of 111 and 201 are poorly defined in the glycosynthase product (Fig. 5). These features of the *in vitro* glycosynthase products, in contrast to the sharp reflections observed for paramylon, are characteristic of crystallites grown two-dimensionally on the $(hk0)$ plane and are related to the crystallite morphology revealed by TEM (Fig. 4).

pH Optima for Glycosynthase Reactions—The pH optima for

the three (1,3)- β -D-glucan glycosynthases were 6–8 (Fig. 6), although the E231G mutant in particular exhibited a broad pH optimum in the range 6–10. These values can be compared with a pH optimum of 4.8 for the wild type (1,3)- β -D-glucan endohydrolase from barley (19, 37). Subsequent kinetic analyses of the mutated enzymes were conducted at pH 6.2.

Comparison of Glycosynthase Activities in Mutants E231G, E231S, and E231A—The rates of formation of (1,3)- β -D-glucan by the mutated enzymes using α -laminaribiosyl fluoride, 3-thio- α -laminaribiosyl fluoride, and 4NPGlc as substrates are compared in Fig. 7. Again, the relatively faster rate of glycosynthase activity is observed with α -laminaribiosyl fluoride and the E231G mutant, compared with the S-glycoside substrate and the E231A and E231S mutants. The rate of E231G glycosynthase action is highest with 4NPGlc (Fig. 7C). The kinetic parameters K_m , k_{cat} , and k_{cat}/K_m of the mutated (1,3)- β -D-glucan endohydrolases, using α -laminaribiosyl fluoride as substrate, are shown in Table I.

Rescue of Activity with Sodium Formate—When the (1,3)- β -D-glucan endohydrolase mutated enzymes E231G, E231A, and E231S were incubated with laminarin, no activity could be detected. However, the addition of sodium formate led to the recovery of up to 75% of wild type activity with the E231G mutant and about 25% of wild type activity with the E231A mutant (Fig. 8). Little or no activity was recovered with the E231S mutant. No activity was recovered with any of the three mutated enzymes following the addition of NaN_3 .

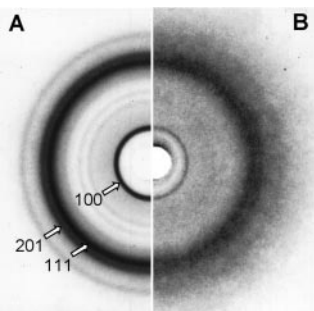


FIG. 5. X-ray diffraction powder diagrams of paramylon and of the product of autocondensation reaction catalyzed by mutated (1,3)- β -D-glucanase E231G. A, paramylon in anhydrous form. The reflections corresponding to the strongest diffraction rings characteristic of paramylon have d spacings of 1.24, 0.45, and 0.421 nm and are indexed as 100, 111, and 201, respectively. B, product synthesized by a mutated E231G (1,3)- β -D-glucanase from α -laminaribiosyl fluoride.

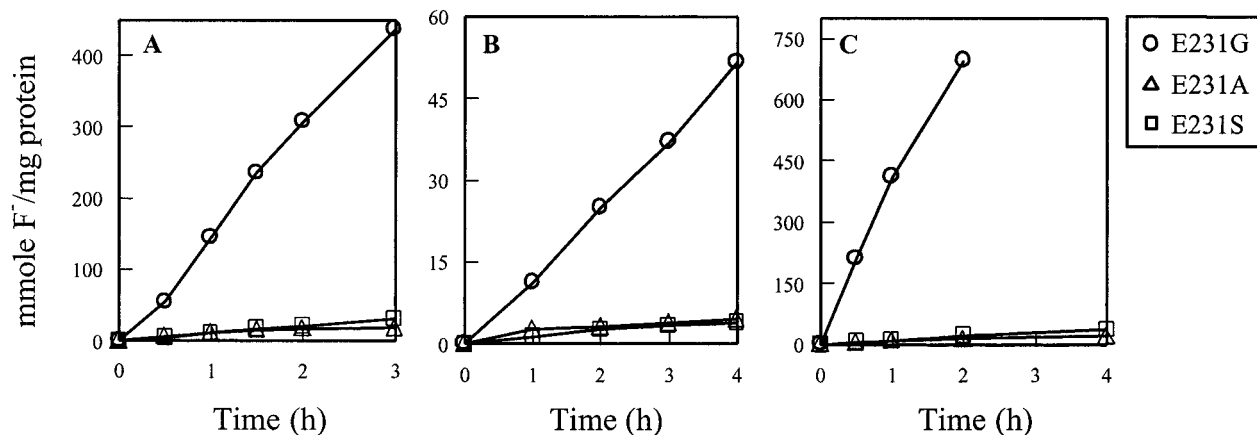


FIG. 7. Time course of autocondensation and heterocondensation reactions, catalyzed by mutated (1,3)- β -D-glucanases E231G, E231A, and E231S. A, 14.7 mM α -laminaribiosyl fluoride; B, 14.7 mM α -thiolaminaribiosyl fluoride; C, 14.7 mM α -laminaribiosyl fluoride and 74 mM 4NPGlc.

DISCUSSION

The codon corresponding to the catalytic nucleophile Glu²³¹ of the barley (1,3)- β -D-glucan endohydrolase isoenzyme GII (17, 37) was altered by site-directed mutagenesis of a nearly full-length cDNA to encode Gly, Ser, or Ala. The mutated cDNAs were expressed in *E. coli* and mutated enzymes were purified by affinity chromatography on nickel-nitrilotriacetic acid columns (Fig. 1). The mutated (1,3)- β -D-glucan endohydrolases had no measurable activity against laminarin, but activity of the E231G and E231A mutants on laminarin could be partially restored through the use of sodium formate as an external nucleophile (Fig. 8). Formate did not restore the activity of the E231S mutant (Fig. 8), probably as a result of steric limitations.

The three mutated enzymes were capable of converting α -laminaribiosyl fluoride into polymeric products that precipitated out of solution during the glycosynthase reaction. The relative rates of precipitation, estimated by visual observation, were E231G \gg E231S $>$ E231A. Given that linear (1,3)- β -D-glucans of DP greater than about 20 are insoluble in aqueous media (38), it was possible that the precipitated material represented (1,3)- β -D-glucan of DP greater than 20. The precipitated reaction products were rapidly hydrolyzed following incubation with wild type (1,3)- β -D-glucan endohydrolase to

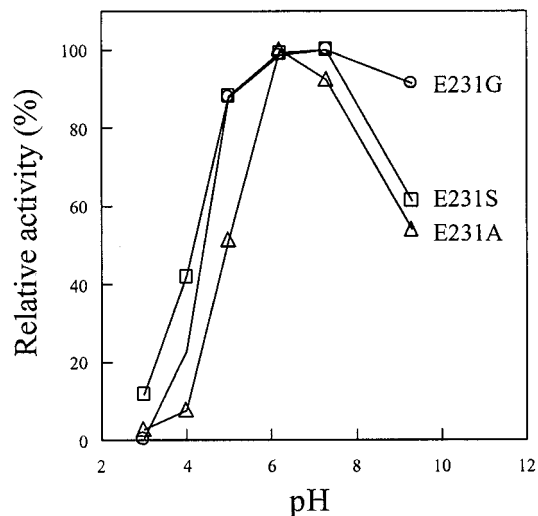


FIG. 6. pH dependence of autocondensation reaction catalyzed by mutated (1,3)- β -D-glucanases E231G, E231A, and E231S with α -laminaribiosyl fluoride.

TABLE I
Kinetic constants of mutated E231G, E231S, and E231A (1,3)- β -D-glucanases

Mutant	$K_m \pm$ S.D. mM	$k_{cat} \pm$ S.D. ^a 10^{-3} s^{-1}	$k_{cat} K_m^{-1b}$ $10^{-4} \text{ M}^{-1} \text{ s}^{-1}$
E231G	9.7 ± 1.2	2.80 ± 0.40	28.9
E231S	19.8 ± 2.2	0.17 ± 0.03	0.9
E231A	25.9 ± 3.2	0.14 ± 0.02	0.5

^a Catalytic rate constant or catalytic center activity.

^b Catalytic efficiency factor.

release major components with mobilities on thin layer chromatograms similar to laminaritriose and laminaribiose (data not shown). Further analysis of the products of the reaction by ^{13}C NMR spectrometry and methylation procedures confirmed that they consisted almost entirely of (1,3)- β -D-glucan (Fig. 2). The methylation analyses suggested that the average DP of the (1,3)- β -D-glucan product was about 30; a similar value was obtained by mass spectrometry.

Thus, the mutated barley (1,3)- β -D-glucan endohydrolases were acting as glycosynthases in aqueous media, using α -laminaribiosyl fluoride as a substrate. Several glycosynthases have been generated by mutagenesis of polysaccharide exohydrolases or glycosidases and used with a variety of β -D-glycoside acceptors to yield mainly low molecular mass oligosaccharides (13, 15). The technology has also been applied to endo-acting enzymes for the synthesis of oligo- and polysaccharides by Malet and Planas (22) and for the synthesis of polysaccharides by Driguez and co-workers (39). A family 16 (1,3;1,4)- β -D-glucan endoglucanase from *Bacillus licheniformis* produces predominantly oligosaccharides (22), as does a family 17 (1,3,1,4)- β -D-glucan endoglucanase from barley.¹ However, a (1,4)- β -D-glucan endoglucanase (endocellulase Cel7B) from *Humicola insolens* has been used in high yielding syntheses of polysaccharides through the formation of (1,4)- β -glucosidic linkages (39). In the case of Cel7B, α -cellobiosyl fluoride was converted in near quantitative yield into cellulose II (39).

Detailed kinetic comparisons of the three barley (1,3)- β -D-glucan glycosynthases with respect to their ability to catalyze the glycosynthase reaction show that catalytic efficiency of the E231G mutant is 30–50 times higher than the E231S and E231A mutants, which exhibit similar values (Table I). Molecular modeling of the E231G mutant, based on the three-dimensional structure of the wild type barley (1,3)- β -D-glucan endohydrolase (17), suggested that replacement of the catalytic nucleophile Glu²³¹ with the much smaller side chain of the Gly residue would leave a relatively deep depression in the floor of the active site cleft immediately adjacent to the catalytic site (Fig. 9). In addition, the negative charge in this region would be reduced (Fig. 9). The most obvious structural explanation for the catalytic efficiency of the E231G mutant is that the depression in the floor of the active site cleft more easily accommodates the axial fluoride atom of the α -laminaribiosyl fluoride substrate without undue interference with the binding of glucosyl residues of acceptor or donor molecules. Mayer *et al.* (40) have also noted that a β -D-glucosidase in which a Gly residue replaces the nucleophile is a relatively efficient glycosynthase.

The kinetic analyses also revealed that pH optima for the glycosynthase reactions were relatively broad, particularly at higher pH values, and were as much as 2–3 pH units higher than the pH optimum of 4.75 observed for the native barley (1,3)- β -D-glucan endohydrolase (Fig. 6; *cf.* Ref. 19). If removal of the catalytic nucleophile causes a downward shift in the $\text{p}K_a$ of the catalytic acid/base (22), which is thereby converted to the ionized, carboxylate form necessary for the catalysis of the glycosynthase reaction (Scheme 2), the steep drop in glycosyn-

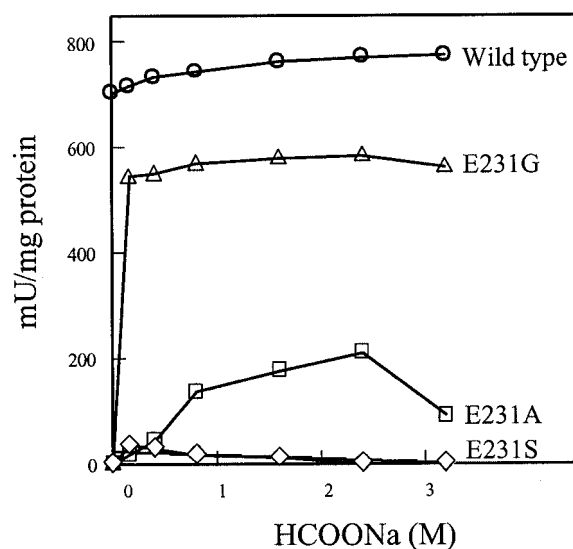


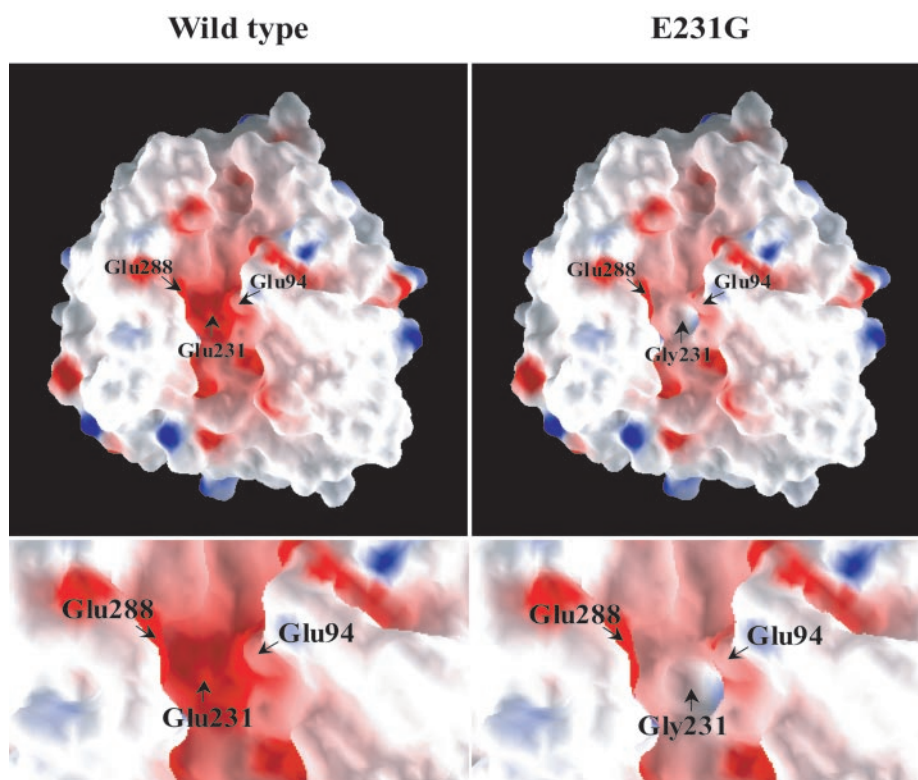
FIG. 8. Hydrolytic activity of the wild type (1,3)- β -D-glucanase and restoration of the hydrolytic activity of mutated (1,3)- β -D-glucanases E231G, E231A, and E231S in the presence of sodium formate. The reaction mixtures, containing laminarin and 0–3.2 M concentrations of sodium formate were incubated with E231G, E231A, and E231S mutated enzymes.

these activity at acidic pH (Fig. 6) could be explained by protonation of that residue. Although early inhibition studies suggested that Glu²⁸⁸ was the catalytic acid/base residue (16), the three-dimensional arrangements of catalytic residues strongly suggest that Glu⁹⁴ is most likely to be the catalytic acid/base residue (41, 42). In addition to Glu⁹⁴ and Glu²⁸⁸, other charged amino acid residues in the immediate vicinity of the catalytic site, including Glu²⁷⁹, Lys²⁸², Arg³¹, Arg¹²⁹, and Arg¹⁷⁴, appear to play a role in catalysis (37). It is therefore difficult to attribute the shape of the pH curve of the glycosynthases (Fig. 6) to specific amino acid residues, but catalytic mechanisms of glycoside hydrolases and glycosynthases described in terms of a single catalytic nucleophile and a single catalytic acid/base might prove to be oversimplified (43).

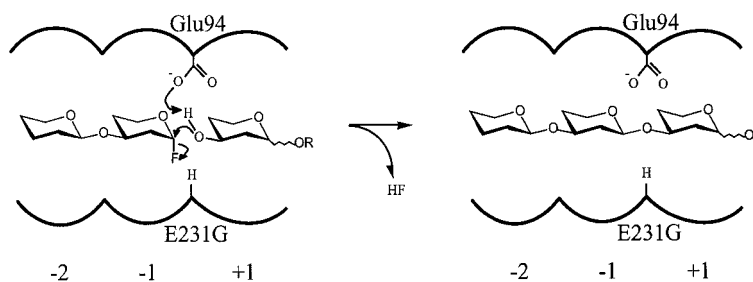
As mentioned above, the products of the glycosynthase reaction catalyzed by the mutated barley enzymes are (1,3)- β -D-glucans with a DP of \sim 30. A key feature of the reaction was that (1,3)- β -D-glucans of very high DP could not be detected, and, in particular, we were unable to detect significant levels of lower molecular mass oligosaccharide products by thin layer chromatography, even when reactions were performed at low temperatures, for short times, or under conditions that were otherwise suboptimal (data not shown). This suggests that the glycosynthase reaction is repetitive, or processive, in the sense that the (1,3)- β -D-glucan product may not be released from the active site of the glycosynthase enzyme until its DP is about 30. It would appear that new glycosynthase product, which spans the catalytic site, slides a distance of two glucosyl-binding subsites toward the acceptor side of the catalytic site and therefore exposes subsites –2 and –1 for binding of the incoming donor molecule (Scheme 2) (44). Numerous aromatic amino acid residues in the substrate-binding cleft of the enzyme (17) may facilitate the movement of the glycosynthase products along the cleft, where a total of eight subsites are known to be present (44). We have no kinetic or structural evidence for the existence of additional substrate-binding sites on the enzyme. It is not known if the native (1,3)- β -D-glucan endohydrolase isoenzyme GII is capable of processive, multiple attacks during hydrolysis, but other endohydrolases do exhibit this property (45, 46).

Transmission and scanning electron microscopy showed that

FIG. 9. Charge distributions mapped onto molecular surfaces of wild type and mutated E231G (1,3)- β -D-glucanases. Top panels show molecular surface morphologies coded by electrostatic potential. Lower panels show details of surface morphologies in the catalytic site regions. The E231G mutation leads to formation of a cavity in the floor of the cleft. Blue and red patches indicate electropositive (24 kiloteslas einstein⁻¹) and electronegative (-18 kiloteslas einstein⁻¹) regions. The drawings were generated using GRASP.



Mechanism of a Glycosynthase Reaction Catalysed by 1,3- β -D-Glucanase E231G



SCHEME 2. Mechanism of a glycosynthase reaction catalyzed by family 17 retaining plant (1,3)- β -D-glucanase E231G. Donor substrate for the glycosynthetic reaction, a reactive α -laminaribiosyl fluoride, mimics an axial α -glycosyl enzyme intermediate, which has been identified as an intermediate in a double displacement reaction mechanism of retaining glycoside hydrolases (48). Catalytic acid/base Glu⁹⁴ in its dissociated form accepts a proton from the equatorial 3-OH group of the acceptor glycoside. This leads to the synthesis of a new (1,3)- β -D-linked glycosidic linkage with a retained anomeric configuration through a glycosyl transfer reaction and a concurrent hydrogen fluoride release. Subsite binding subsites -2, -1, and +1 are marked.

the (1,3)- β -D-glucan products of the glycosynthase reaction formed crystalline hexagonally shaped platelets (Figs. 3 and 4). Closer examination of the crystals (Fig. 4A) reveals platelets may grow on previously formed crystallites in a screwlike manner, as previously reported for recrystallized curdlan (34). It is noteworthy that the barley glycosynthase generates these crystallite structures at 30 °C, whereas recrystallization by hydrothermal treatment is normally required for their formation. Comparison of x-ray diffraction patterns with the well characterized (1,3)- β -D-glucan, paramylon, indicated that the (1,3)- β -D-glucan product of the barley glycosynthase adopts a triple helical conformation within these crystallites (Fig. 4B). The electron diffraction patterns (Fig. 4A, inset) further suggested that the triple helices were oriented perpendicularly to the

plane of the crystallite, as previously shown for recrystallized curdlan (34). Estimates of the DP of the (1,3)- β -D-glucan products, based on these dimensions of the crystallite, corresponded closely to the DP of about 30 estimated by mass spectrometry and methylation analyses.

The narrow range of DP values observed for the glycosynthase products may result from the dissociation of the products from the enzyme at a DP where (1,3)- β -D-glucans become insoluble or form triple helices; both occur at a DP of 20–30 (5, 38). In any case, the relatively large, hexagonal crystallite structures of (1,3)- β -D-glucans generated by the barley glycosynthase contrast sharply with products of membrane-bound (1,3)- β -D-glucan synthases, where very high DP but relatively narrow, microfibrillar (1,3)- β -D-glucans are formed (28, 47).

Whereas the extent of the (1,3)- β -D-glucan polymerization reaction catalyzed by the glycosynthase might be limited by product entanglement and precipitation, membrane-bound (1,3)- β -D-glucan synthase complexes have clearly evolved an ability to generate much longer, microfibrillar polysaccharides.

Finally, it has been demonstrated here that the barley (1,3)- β -D-glucan glycosynthase E231G exhibits some flexibility with respect to its substrate for the condensation reaction. In addition to α -laminaribiosyl fluoride, 3-thio- α -laminaribiosyl fluoride could also be polymerized, although the latter reaction occurred at a slower rate, and the products were of lower DP and, in some cases, cyclic (data not shown). This indicates that the glycosynthase could be adapted or fine tuned for the generation of cyclic (1,3)- β -D-glucans or for the synthesis of (1,3)- β -D-glucans with alternating *S*- and *O*-glycosidic linkages. More detailed analyses of donor and acceptor specificity of the E231G mutant have now shown that the barley (1,3)- β -D-glucan glycosynthase can catalyze the condensation of various β -D-glucosides, β -D-xylosides, and β -D-galactosides.³ This raises the possibility that the barley glycosynthase could prove useful for the rapid and facile biosynthesis, in high yields and at low cost, of complex oligosaccharides with specified monosaccharide sequences, substitution patterns, and branching characteristics. Such polysaccharides or oligosaccharides could find pharmaceutical or medical applications in the future.

Acknowledgments—Dr. C. Bosso, Dr. J. Hayasaka, Dr. G. Currie, and S. Fruchard are thanked for technical assistance. We thank Professor Bruce Stone and Dr. Henri Chanzy for valuable assistance with aspects of the work.

REFERENCES

- Estrada, A., Yun, C-H., Van Kessel, A., Li, B. Hauta, S., and Laarveld, B. (1997) *Microbiol. Immunol.* **41**, 991–998
- Ohno, N., Miura, N. N., Nakajima, M., and Yadomae, T. (2000) *Biol. Pharmacol. Bull.* **23**, 866–872
- Maeda, Y. Y., and Chihara, G. (1971) *Nature* **229**, 634
- Wasser, S. P., and Weis, A. L. (1999) *Crit. Rev. Immunol.* **19**, 65–96
- Stone, B. A., and Clarke, A. E. (1992) *The Chemistry and Biology of (1,3)- β -Glucans*, La Trobe University Press, Bundoora, Victoria, Australia
- Falch, B. H., Espevik, T., Ryan, L., and Stokke, B. T. (2000) *Carbohydr. Res.* **329**, 587–596
- McCarter, J. D., and Withers, S. G. (1994) *Curr. Opin. Struct. Biol.* **4**, 885–892
- Armand, S., Vieille, C., Gey, C., Heyraud, A., Zeikus, J. G., and Henrissat, B. (1996) *Eur. J. Biochem.* **236**, 706–713
- Armand, S., Drouillard, S., Schüle, M., Henrissat, B., and Driguez, H. (1997) *J. Biol. Chem.* **272**, 2709–2713
- Planas, A. (2000) *Biochim. Biophys. Acta* **1543**, 361–382
- Biely, P., Vrsanska, M., and Kratky, Z. (1980) *Eur. J. Biochem.* **112**, 375–381
- Hrmova, M., Fincher, G. B., Viladot, J. L., Planas, A., and Driguez, H. (1998) *J. Chem. Soc. Perkin Trans. 1*, 3571–3576
- Mackenzie, L. F., Wang, Q., Warren, R. A. J., and Withers, S. G. (1998) *J. Am. Chem. Soc.* **120**, 5583–5584
- Trincon, A., Perugino, G., Rossi, M., and Moracci, M. (2000) *Biorg. Med. Chem. Lett.* **10**, 365–368
- Nashiru, O., Zechel, D. L., Stoll, D., Mohammadzadeh, T., Warren, R. A. J., and Withers, S. G. (2001) *Angew. Chem. Int. Ed.* **40**, 417–420
- Chen, L., Fincher, G. B., and Høj, P. B. (1993) *J. Biol. Chem.* **268**, 13318–13326
- Varghese, J. N., Garrett, T. P. J., Colman, P. M., Chen, L., Høj, P. B., and Fincher, G. B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 2785–2789
- Stewart, R., Varghese, J. N., Garrett, T. P. J., Høj, P. B., and Fincher, G. B. (2001) *Protein Eng.* **14**, 245–253
- Hrmova, M., and Fincher, G. B. (1993) *Biochem. J.* **289**, 453–461
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
- Hrmova, M., Harvey, A. J., Wang, J., Shirley, N. J., Jones, G. P., Stone, B. A., Høj, P. B., and Fincher, G. B. (1996) *J. Biol. Chem.* **271**, 5277–5286
- Malet, C., and Planas, A. (1998) *FEBS Lett.* **440**, 208–212
- Contour-Galcerà, M.-O., Guillot, J.-M., Ortiz-Mellet, C., Pfeiffer-Carrara, F., Defaye, J., and Gelas, J. (1996) *Carbohydr. Res.* **281**, 99–118
- Samuels, M. L. (1991) *Statistics for Life Sciences*, Dellen Publishing Co., San Francisco
- Perella, F. (1988) *Anal. Biochem.* **174**, 437–447
- Harris, P. J., Henry, R. J., Blakeney, A. B., and Stone, B. A. (1984) *Carbohydr. Res.* **127**, 59–73
- Bulone, V., Fincher, G. B., and Stone, B. A. (1995) *Plant J.* **8**, 213–225
- Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. Sect. A* **47**, 110–119
- Sali, A., and Blundell, T. L. (1993) *J. Mol. Biol.* **234**, 779–815
- Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* **18**, 2714–2723
- Nicolls, A., Sharp, K., and Hönig, B. (1991) *Proteins* **4**, 281–296
- Høj, P. B., Hartman, D. J., Morrice, N. A., Doan, D. N. P., and Fincher, G. B. (1989) *Plant Mol. Biol.* **13**, 31–42
- Saito, H., Tabeta, R., and Harada, T. (1981) *Chem. Lett.* 571–574
- Chanzy, H., and Vuong, R. (1985) *Polysaccharides: Topics in Structure and Morphology* (Atkins, E. D. T., ed) The Macmillan Press, Hampshire, UK
- Deslandes, Y., Marchessault, R. H., and Sarko, A. (1980) *Macromolecules* **13**, 1466–1471
- Chuah, T. C., Sarko, A., Deslandes, Y., and Marchessault, R. H. (1983) *Macromolecules* **16**, 1375–1382
- Chen, L., Garrett, T. P. J., Fincher, G. B., and Høj, P. B. (1995) *J. Biol. Chem.* **270**, 8093–8101
- Harada, T. (1977) *Am. Chem. Soc. Symp. Ser.* **45**, 265–283
- Fort, S., Boyer, V., Greffe, L., Davies, G. J., Moroz, O., Christiansen, L., Schüle, M., Cottaz, S., and Driguez, H. (2000) *J. Am. Chem. Soc.* **122**, 5429–5437
- Meyer, C., Zechel, D. L., Reid, S. P., Warren, A. J., and Withers, S. G. (2000) *FEBS Lett.* **466**, 40–44
- Henrissat, B., Callebaut, I., Fabrega, S., Lehn, P., Mornon, J. P., and Davies, G. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7090–7094
- Jenkins, J., Lo Leggio, L., Harris, G., and Pickersgill, R. (1995) *FEBS Lett.* **362**, 281–285
- Joshi, M. D., Sidhu, G., Nielsen, J. E., Brayer, G. D., Withers, S. G., and McIntosh, L. P. (2001) *Biochemistry* **40**, 10115–10139
- Hrmova, M., Garrett, T. P. J., and Fincher, G. B. (1995) *J. Biol. Chem.* **270**, 14556–14563
- Robyt, J. F., and French, D. (1967) *Arch. Biochem. Biophys.* **122**, 8–16
- Parsiegla, G., Reverbel-Leroy, C., Tardif, C., Belaich, J.-P., Driguez, H., and Haser, H. (2000) *Biochemistry* **39**, 11238–11246
- Jabri, E., Quigley, D. R., Alders, M., Hrmova, M., Taft, C. S., Phelps, P., and Selitrennikoff, C. P. (1989) *Curr. Microbiol.* **12**, 153–162
- Zechel, D. L., and Withers, S. G. (2000) *Acc. Chem. Res.* **33**, 11–18

³ J. K. Fairweather, S. J. Rutten, M. Hrmova, G. B. Fincher, and H. Driguez, unpublished data.