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Synthesis of Cyclic β-Glucan Using Laminarinase 16A Glycosynthase Mutant from the Basidiomycete *Phanerochaete chrysosporium*

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Abstract: Glycosynthases are precise molecular instruments for making specifically linked oligosaccharides. X-ray crystallography screening of ligands bound to the 1,3(4)-β-D-glucanase nucleophile mutant E115S of *Phanerochaete chrysosporium* Laminarinase 16A (Lam16A) showed that laminariheptaose (L7) bound in an arch with the reducing and nonreducing ends occupying either side of the catalytic cleft of the enzyme. The X-ray structure of Lam16A E115S in complex with α-laminariheptaosyl fluoride (αL7F) revealed how αL7F could make a nucleophilic attack upon itself. Indeed, when Lam16A E115S was allowed to react with αL7F the major product was a cyclic β-1,3-heptaglucan, as shown by mass spectrometry. NMR confirmed uniquely β-1,3-linkages and no reducing end. Molecular dynamics simulations indicate that the cyclic laminariheptaose molecule is not completely planar and that torsion angles at the glycosidic linkages fluctuate between two energy minima. This is the first report of a glycosynthase that joins the reducing and nonreducing ends of a single oligosaccharide and the first reported synthesis of cyclic β-glucan.

1. Introduction

The chemical synthesis of specific oligosaccharides has traditionally been extremely difficult. By harnessing the regio- and stereoselectivity of glycoside hydrolases [1−3] however, scientists have been able to overcome many of these difficulties. Glycosynthase mutants of retaining glycoside hydrolases, where the catalytic nucleophile residue has been replaced by a smaller amino acid residue (commonly serine, alanine or glycine), are utilized together with substrates where the anomeric oxygen of the substrate is replaced by an electronegative leaving group (such as a fluoride[4]) in the anomeric position. The α-glycosyl fluoride bound in a nucleophile mutant mimics the glycosyl-enzyme intermediate of the wild-type retaining glycoside hydrolase[5] with the partially charged anomeric carbon atom at the −1 subsite exposed to nucleophilic attack.[6] In a retaining glycoside hydrolysis reaction, the oxygen of water usually carries out the attack. For a glycosynthase reaction, by contrast, the closest hydroxyl group at the +1 subsite attacks the anomeric carbon leading to the formation of a new glycosidic bond.

Apart from their use in glycosynthase reactions,[7] nucleophile mutants can be used as analytical tools for making snapshots of a reaction.[5] With a disabled nucleophile, natural ligands can bind in the active site as they would prior to hydrolysis in the nonmutated wild-type enzyme. Because of the missing nucleophile, however, the hydrolysis normally catalyzed by the wild-type enzyme does not take place in the nucleophile mutant. X-ray crystallography can then be used to make snapshots of such "frozen moments".

We made such nucleophile mutations in *Phanerochaete chrysosporium* laminarinase 16A (Lam16A)[8] for determination of structures with substrates spanning the active site. Surpris-

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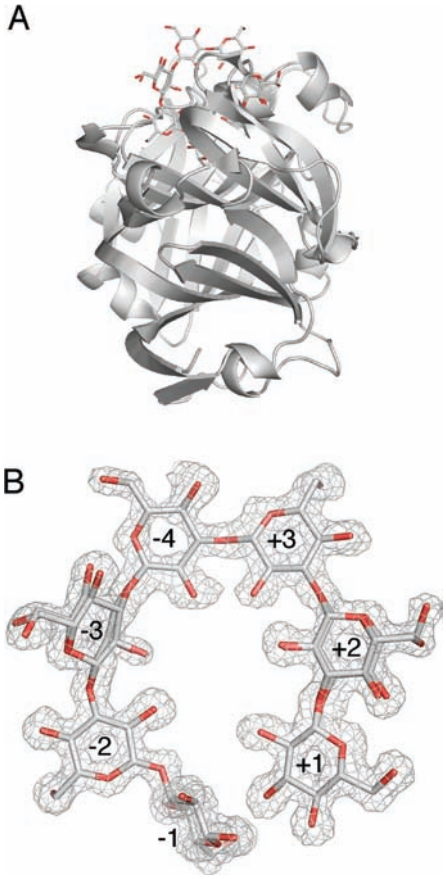

Figure 1. (A) L7 and αL7F ligands form an arch when bound to Lam16A E115S enzyme with reducing and nonreducing ends almost joining in the nucleophile-depleted catalytic cleft. (B) The bound L7 ligand with electron density contoured at 0.42 e Å^{-3}. Double conformations were refined for the −3 and +2 glucose residues.

ingly, the structure of the Lam16A nucleophile variant E115S, in complex with β-1,3-glucan laminariheptaose, revealed that the bound oligosaccharide "bent back upon itself", forming a loop with seven glucosyl residues with the ends meeting at the catalytic center of the enzyme. Inspired by these results, we synthesized α-laminariheptaosyl fluoride (αL7F) to test whether the ends of the ligand may be joined via a glycosynthase reaction. A cyclic heptaglucan was indeed formed upon reaction with the Lam16A E115S variant, as confirmed by matrix-assissted laser desorption/ionization (MALDI) MS and NMR spectroscopy.

2. Results

This paper includes three main experiments: (1) the elucidation (by X-ray crystallography) of unmodified laminariheptaose (L7) and αL7F bound in the catalytic site of the Lam16A E115S variant, (2) a time study of Lam16A E115S reacting with αL7F, and (3) MS and NMR analysis of the cyclic L7 product from this reaction, as well as molecular dynamics simulations of open-chain and cyclic L7 (cL7).

2.1. X-ray Crystallography of Lam16A E115S in Complex with L7 and αL7F. Both L7 and αL7F structures reveal a β-1,3-heptaglucan that binds in an arch (Figure 1), protruding from the catalytic center and binding simultaneously in the donor and acceptor sites of Lam16A E115S. The only significant difference between the two β-glucan ligands is the position of the atoms at the anomeric center in subsite −1 (C1, β-O/α-F;

Figure 2. Close-up superposition of the L7 (white) and αL7F (gray) structures of Lam16A E115S mutant showing the positions of the −1 and +1 glucose residues and the catalytic acid/base Glu120 (E120). The +1 glucose residue is in proper orientation for the formation of a β-1,3 glycosidic bond in the αL7F structure.

Table 1. X-ray Diffraction Data and Refinement Statistics for the Lam16A E115S Structures

PDB code	2wlq	2wne
ligand	L7	αL7F
	Data Collection	
beamline[a]	I911-3	I911-2
wavelength [Å]	0.98	1.04
space group	$P2_12_12_1$	$P2_12_12_1$
cell parameters [Å]	38.4 × 47.9 × 152.4	38.2 × 47.9 × 152.8
resolution range [Å]	25.0−1.40	40.6−2.12
resolution range outer shell [Å]	1.48−1.40	2.22−2.12
no. unique reflections	56 249	16 720
multiplicity	6.3 (4.4)	3.6 (3.3)
completeness [%]	99.8 (98.2)	96.9 (80.9)
R_{merge} [%][b]	8.5 (39.6)	8.7 (23.7)
$\langle I/\sigma(I)\rangle$	16.6 (3.3)	12.0 (4.9)
	Structure Refinement	
R_{final}/R_{free} [%]	16.4/19.4	16.0/22.0
no. atoms	2835	2693
protein (B_{avg} [Å^2]), chain A (1−298)	2275 (10.2)	2269 (10.6)
ligand (B_{avg} [Å^2]), chain B (1401−7)	100 (17.6)	78 (25.2)
water (B_{avg} [Å^2]), chain W	363 (25.8)	263 (23.5)
glycosylation (B_{avg} [Å^2]), chain A	97 (25.8)	83 (35.6)
rms bond length [Å]	0.013	0.014
rms bond angles [deg]	1.48	1.48
Ramachandran plot outliers[c]	T81,T151,C236, D243,C254,W257	T81,T151,D243, C254,W257

[a] MaxLab, Lund, Sweden. [b] $R_{merge} = \Sigma hkl\Sigma i\ |Ihkli - \langle Ihkl\rangle|/\Sigma hkl\Sigma i\langle Ihkl\rangle$. [c] φ and ψ peptide dihedral outliers[11] as defined by AutoDep.[12]

Figure 2). Statistics from X-ray diffraction data collection and structural refinement are summarized in Table 1. Table 2 lists protein/ligand interactions, and Table 3 lists dihedral angles between glucose units.

The two protein structures are very similar to each other and to previous structures of Lam16A (normalized[9] rmsd <0.2 Å). Two of the amino acids warrant a comment: Asn162 and Trp257.

Asn162 is the amino acid within the mobile loop of residues 159−162. In an earlier study of the wild-type Lam16A/G4G3G complex[8] (PDB code 2W39), Asn162 is hydrogen-bonded to the −1 glucose. For the bound L7 and αL7F, however, Asn162 forms a hydrogen bond with the hydroxyl group O2 of the glycoside at subsite +2 (Table 2). In other words, Asn162 is

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Table 2. Hydrogen-Bonding and Hydrophobic (Trp 103, 110, 257) Interactions between Bound L7 Ligand and Lam16A E115S Nucleophile Mutant (PDB code 2wlq) (Water-Mediated H-Bonds Not Shown)

subsite	protein/ligand interactions
+1	Trp257
	O3-Glu120 Oε2
	O4-Glu120 Oε1, -Asp117 Oδ2,
	-His133 Nε2
	O6-Thr163
+2	Trp257
	O2-Asn162 Nδ2
	O5-Gln260 Nε2
+3	O3-Gln260 Nε2, -Asp256 O
−4	O2-Asp256 Oδ2
−3	O2-Pro26 O
−2	Trp110
	O6-Arg73 Nη2
−1	Trp103
	O1-His133 Nε2, -Asp117 Oδ2, -Asp117 Oδ1
	O5-Glu120 Oε2, -Asp117 Oδ2, -Asp117 Oδ1
	O6-Glu120 Oε2, -Trp99 Nε1

Table 3. Dihedral φ and ψ Angles of Glycosidic Linkages of L7 Ligand in Lam16A E115S Nucleophile Mutant (2wlq)[a] and of Free Energy Minima for L7 (Open) and cL7 (Cyclic) Laminariheptaose Models from MD Simulations[b]

subsite	φ O5′−C1′−O3−C3	ψ C1′−O3−C3−C2
+1/+**2**	−89	−149
+2/+**3**	−119	−116
+3/−**4**	−127	−126
−4/−**3**	−121	−151
−3/−**2**	−101	−98
−2/−**1**	−66	−93
L7 model	−80	−100
cL7 model (A)	−95	−105
cL7 model (B)	−140	−140

[a] A-conformers listed, as B-conformers differ in the positioning of O6 only. [b] Note two minima, A and B, for cL7.

now hydrogen-bonding to an acceptor-site glycoside instead of bonding to a donor-site glycoside.

One of the Ramachandran plot outliers in the two structures is Trp257 (Table 1). This residue was also an outlier for the previously presented (wild-type) Lam16A structure with a ligand in the acceptor site (PDB code 2W52).[10] Although the stacking of Trp257 with +1/+2 subsite glucose residues is imperfect, the position of Trp257 could be critical in binding the acceptor.

2.2. Time Study of E115A Lam16A Reacting with αL7F. Reversed-phase chromatography (RPC) analysis of the reaction solution (30 °C) at start and after 4 and 48 h revealed the emergence of a prominent new peak (Figure 3) after 4 h. Initially, the major peak is the substrate, αL7F, at the 12.1 min elution time. For the 4 h sample, another prominent peak emerged, at the 17.7 min elution time. The 48 h sample did not show any presence of the αL7F peak.

2.3. MS and NMR Analysis of the Cyclic L7 Product. The MALDI mass spectrogram of the reaction product (Figure 4) unambiguously showed only two peaks: *m/z* 1157.37 for the Na^+ adduct and *m/z* 1173.32 for the K^+ adduct, each grouped together with their smaller and characteristic isotopic sister

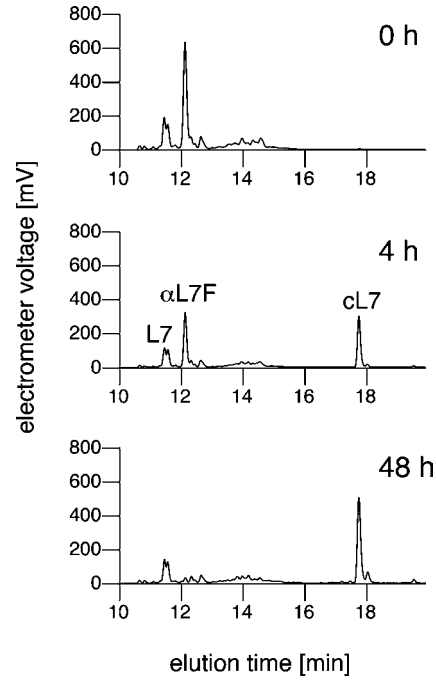

Figure 3. Three snapshots (0, 4, 48 h) of the Lam16A E115S/αL7F glycosynthase reaction using charged aerosol detection (CAD) of products eluted from a C18 column with an acetonitrile gradient. At 0 h reaction time, the prominent peak is the substrate, α-laminariheptaosyl fluoride (αL7F), eluted at 12.1 min. Preceding it is laminariheptaose, L7. After 4 h of reaction with the Lam16A E115S mutant, another prominent peak emerged at the 17.7 min elution time (cL7). By 48 h, the αL7F peak had disappeared.

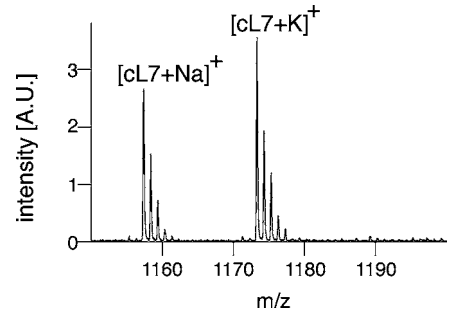

Figure 4. MALDI-MS. Cyclic heptaose product showing two peaks (with their isotope sister peaks) from the mass spectrogram of the 48 h reaction product: *m/z* 1157.37 for the Na^+ adduct and *m/z* 1173.32 for the K^+ adduct. Note the absence of peaks at 1175 and 1191, i.e. where the adduct peaks for noncyclic L7 would appear.

peaks. Subtraction of the masses of the omnipresent Na^+ and K^+ ions from the singly charged products revealed 1134 g/mol as the mass of the product. This is the mass of a heptaglucan without a reducing end, $(C_6H_{10}O_5)_7$, or, in other words, of a cyclic heptaglucan. The calculated values ($m(C_6H_{10}O_5)_7$ + $m(Na^+)$ = (162.05 × 7) + 22.99 = 1157.36; $m(C_6H_{10}O_5)_7$ + $m(K^+)$ = (162.05 × 7) + 38.96 = 1173.33 match those of the observations (see above).

NMR analysis gave only a single set of resonances in the ^1H,^{13}C-HSQC (heteronuclear single quantum coherence) spectrum (Figure 5; Table 4), demonstrating that the isolated cL7 product consists of only glucosyl residues, all connected by the same type of glycosidic linkages without any reducing end, hence confirming that it must be cyclic. The D-glucosyl residues are β-linked because (1) the $J_{H1,H2}$ coupling constant of 7.8 Hz for D-glucopyranose is characteristic of a β-anomer (∼8 Hz)

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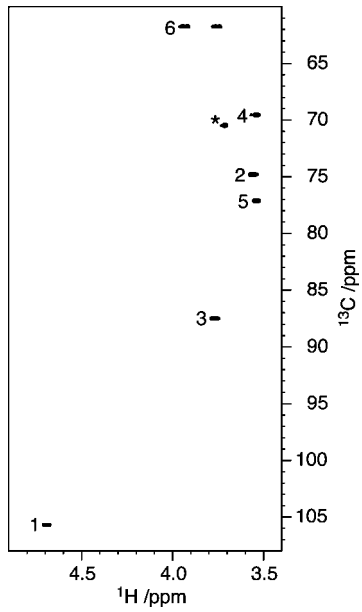

Figure 5. ^{1}H ^{13}C-HSQC NMR spectrum at 70 °C of the cyclic laminariheptaose product. The cross-peaks are labeled by atom position. The cross-peak at δ_H/δ_C 3.71/70.42 marked with an asterisk results from a polyethylene glycol impurity.

Table 4. ^{1}H and ^{13}C NMR Chemical Shifts (ppm) at 70 °C of the Resonances from Cyclic Laminariheptaose (cf. Figure 5)[a]

	1	2	3	4	5	6
^{1}H	4.68 [7.8]	3.56	3.77	3.54	3.53	3.75, 3.93
$\Delta\delta_H$	(−0.06)	(−0.02)	(−0.01)	(0.01)	(0.01)	(0.01), (0.01)
^{13}C	105.67	74.76	87.44	69.51	77.09	61.69
$\Delta\delta_C$	(2.30)	(0.84)	(1.70)	(0.40)	(0.51)	(−0.01)

[a] $J_{H1,H2}$ is given in square brackets, and chemical shift differences compared to Curdlan (as predicted by the program CASPER[16]) are given in parentheses.

and not of an α-anomer (∼4 Hz)[13−15] and (2) the H1 proton chemical shift of 4.68 ppm is characteristic of β-linkages in hexopyranose oligosaccharides.[13−15] The ^{1}H and ^{13}C chemical shifts obtained from the cross-peaks in the NMR spectrum (Figure 5; Table 4) agree well with those for Curdlan, a β-(1→3)-glucan, predicted by the computer program CASPER,[16] providing evidence that all glycosidic linkages were β-(1→3). Interestingly, the ^{13}C chemical shift differences for C1 and C3 deviate by ∼2 ppm, and since these two carbon atoms are part of the glycosidic linkage, this alteration indicates some change in average conformation for glycosidic linkages of the cyclic heptasaccharide compared to Curdlan (Table 4).

In addition a DOSY NMR experiment[17] was employed to investigate the translational diffusion of cL7 and the impurity which has δ_H/δ_C 3.71/70.42. The translational diffusion coefficient of the impurity was lower, indicating a larger molecular mass, than that of the cylic heptaglucan thereby confirming that the compounds are different entities.

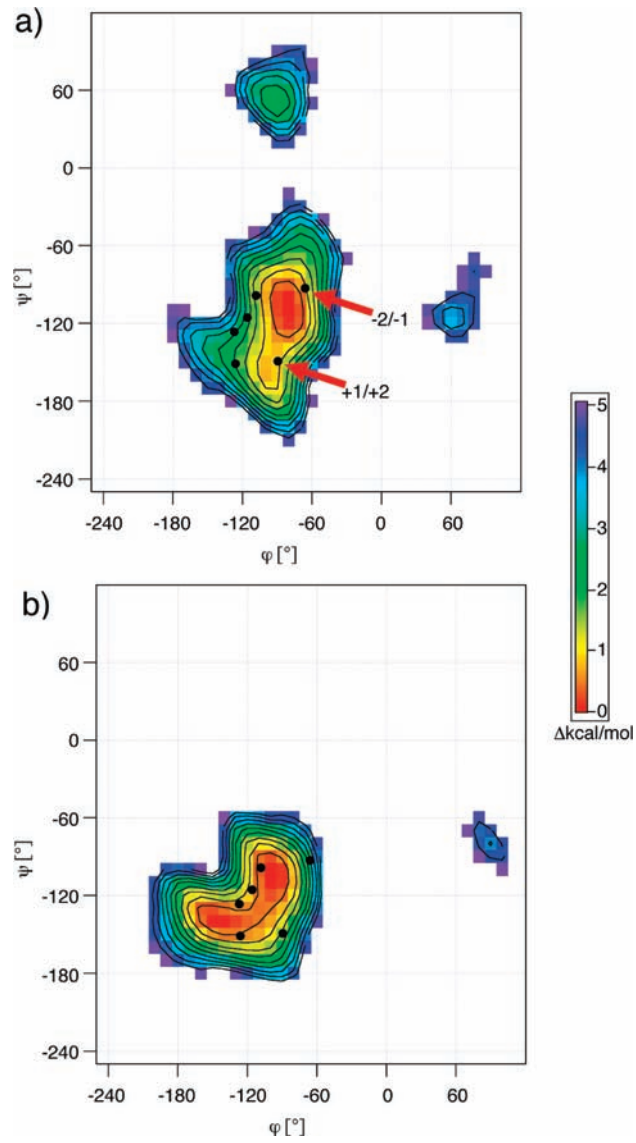

Figure 6. Glycosidic torsion angles of the crystalized ligand (when in complex with Lam16A E115S) plotted as black dots onto free energy maps of both L7 (a) and cL7 (b) derived from 100 ns MD simulations using TINKER/MM3. Note two minima for cL7 (b). The arrows (a) point to the −2/−1 and +1/+2 linkages at the donor and acceptor ends of the bound heptaglucan.

In summary, both MS and NMR results exclude the possibility of any reducing end in the formed product; hence it has to be a cyclic molecule. The mass spectrum showed that the product contained seven unmodified hexose (glucose) units. The NMR spectra revealed that these units had to be β-(1→3)-linked in series, disqualifying the possibility of any branching or alternate glycosynthase reactions other than that of Scheme 1.

2.4. Molecular Dynamics Simulations of L7 and cL7. Molecular dynamics (MD) simulations of open-chain (L7) and cyclic laminariheptaose (cL7) were performed, and free energy conformational maps of the glycosidic torsion angles[18] ϕ and ψ were calculated (Figure 6). A global energy minimum was obtained for L7, at ϕ/ψ torsion angles −80°/−100° (Table 2, Figure 6), indicating that a helical conformation (pitch 22 Å, 6 residues per turn) is most favorable (as depicted for αL7F in

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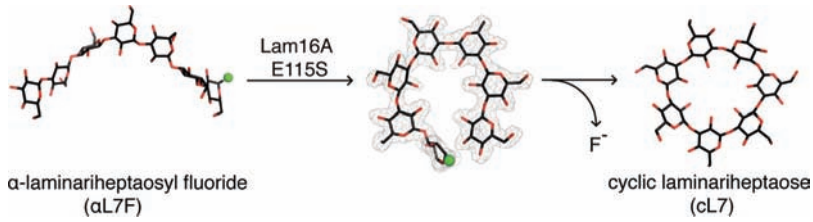
Scheme 1



Scheme 1). With cyclization, two separate energy minima of approximately −95°/−105° and −140°/−140° (Table 2, Figure 6) replace the original minimum, and glycosidic linkages fluctuate between these two minima. The resulting conformation of the cL7 molecule is rather flat (Scheme 1) but not completely planar. The difference in conformational minima between linear L7 and cyclic cL7 could explain the ^{13}C chemical shift differences for C1 and C3 in the NMR analysis mentioned above. The fluctuations between the cL7 minima (that are separated by a energy barrier of ∼0.5 kcal/mol) also explain why only one set of chemical shifts have been found.[19]

For comparison, the torsion angles of the glycosidic linkages in the enzyme-bound L7 ligand (Table 3) were plotted onto the MD free energy maps (Figure 6). All six glycosidic ϕ/ψ torsions are located outside the 1 kcal/mol energy contour of the L7 conformational energy map indicating that conformational energy is required for L7 to adopt the enzyme-bound arch conformation. A comparison with the conformational map of cL7 shows that the ϕ/ψ torsions of all linkages are located inside or close to the 1 kcal/mol energy contour except the linkages at the two ends of the chain.

3. Discussion

α-Glycosyl fluorides act as donor sugars in nucleophile-crippled mutants of retaining glycoside hydrolases because they mimic the glycosyl-enzyme intermediate of the wild-type enzyme.[20] The net result of the reaction is dependent on which acceptors are able to attack the anomeric carbon at the reducing end the fastest. αL7F incubated with Lam16A E115S may thus produce three possible reactions: (i) Hydrolysis, when a water molecule acts as an acceptor as in the wild-type enzyme, giving L7 as product. (ii) Cyclization, when the nonreducing end of the same αL7F molecule acts as an acceptor, yielding cL7. (iii) Elongation, when the nonreducing end hydroxyl of another αL7F molecule acts as an acceptor, yielding αL14F, which may in turn be elongated, and so on. If the elongation reaction is highly preferred it should eventually lead to the formation of an insoluble precipitate. Furthermore (iv), the αL7F preparation contained a small amount of nonderivatized (unmodified) L7 that could have acted as an acceptor and resulted in formation of L14.

The only peak in the RPC chromatograms that decreased significantly in size during the course of the reaction was that of αL7F (which would rule out scenario iv). The αL7F peak had essentially disappeared after 48 h, showing that the compound was completely consumed. No precipitation of the ligand was observed, ruling out the formation of insoluble elongation products (iii). One major new peak appeared during the reaction, corresponding to cyclic L7 (ii), as confirmed by MALDI-MS (Figure 4) and NMR (Figure 5). Formation of other peaks was insignificant compared to that of the cL7 product (Figure 3). No hydrolysis of the α-fluoride (i) could be detected, since there was no increase in the size of the L7 peak (the would-be product of such a hydrolysis).

The Lam16A E115S mutant catalyzes the cyclization of αL7F into cL7 in a nearly quantitative yield. The cyclization reaction (ii) is obviously highly preferred over other plausible outcomes (i, iii, iv), suggesting that the arched αL7F fills the +1 subsite at approximately the same time as the α-glucosyl fluoride unit occupies the −1 subsite, excluding any other acceptor candidates from the glycosynthase reaction. Note that the affinity for the nonreducing (acceptor) end of the same αL7F molecule does not need to be higher than that of other acceptors. Once the reducing end of the donor molecule is bound, the diffusion of the acceptor end is strictly limited, making its effective (local) concentration extremely high compared to other acceptors, which are thus effectively outcompeted at the acceptor site. One alternative acceptor that might still compete is water, but water tends to be a poor acceptor in nucleophile-crippled mutants.[20,21]

Although earlier researchers have reported the cyclization of carbohydrates by unmodified enzymes,[22−24] this is the first report of a glycosynthase ligating the reducing and nonreducing ends of a single modified (αL7F) substrate. The cyclic product (cL7), described here for the first time, has the same amount of glucose units as the substrate. The inherent curvature of β-1,3-glucans[25] with a helical repeat of six glucose residues[10,26,27] is key to this reaction. The MD simulations show that the mutant enzyme does not dramatically force the curvature upon the ligand. Rather, the enzyme simply stabilizes the ends of a helical substrate (as evidenced by lower *B*-factors of the end residues), freezing the helical substrate in an interrupted circle (Figure 1). By applying the principles of glycosynthase reactions, we have been able to exploit the interrupted circle conformation and join the reducing and nonreducing ends to form a cyclic heptaglucan.

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4. Materials and Methods

4.1. Site-Directed Mutagenesis and Protein Preparation. Site-directed mutagenesis by the overlap extension method[28] was used to obtain both E115S and E120A (to be used in future experiments) mutants. The oligonucleotide primers, E115S-F, 5′-CCCAATGGGGGCTCAGTGGACATCATCGAG-3′; E120A-R, 5′-GTCGTTGACGCCCGCGATGATGTCCAC-3′ (mutated nucleotides are underlined); AP1-*Eco*RI-F, 5′-TTTTCAGCGTTCTCGGAATTCGCCACCTACCACCTCGAAGACAAT-3′;AP1-F,TTTTCAGCGTTCTCGGAATTC-3′; AP2-*Not*I-R, 5′-TTACAGTAATATAAAGAATTTGCGGCCGCTCACTGGTAGACCCGGACCGAGGC-3′; AP2-R, 5′-TTACAGTAATATAAAGAATTTGCGGCCGC-3′, were used to obtain the nucleotide fragments of E115S and E120A. The fragments were subcloned into the PCR 4Blunt-TOPO vector (Invitrogen, Carlsbad, CA), and the vector was digested with *Eco*RI and *Not*I, which were followed by ligation into the expression vector pPICZαA (Invitrogen). The E115S-pPICZαA vector was first linearized with the restriction enzyme *Bpu*1102I and then transformed into the yeast *Pichia pastoris* strain KM71H by electroporation. The Zeocin-resistant transformant was cultivated in a growth medium (10 g/L yeast extract, 20 g/L peptone, and 10 mL/L glycerol) for 24 h at 28 °C, then in induction medium (10 g/L yeast extract, 20 g/L peptone, and 10 mL/L methanol) for 48 h at 26 °C, with addition of another 10 mL/L methanol after 24 h.

Crude enzyme solutions were precipitated from culture supernatant with ammonium sulfate (70% saturation). These solutions were applied to an anion exchange (AIEX) Source 30Q column (GE Healthcare, Uppsala, Sweden) equilibrated with 25 mM Bis-Tris buffer, pH 6.2. Bound protein was then eluted with a linear 0−0.5 M NaCl gradient. Peak fractions were concentrated and applied to a (HIC) Phenyl Sepharose 6 column (GE Healthcare, Uppsala, Sweden) equilibrated with 25 mM Bis-Tris buffer and 1 M ammonium sulfate, pH 6.2. The fractions containing recombinant proteins were further purified on an AIEX Mono Q HR 5/5 column (GE Healthcare, Uppsala, Sweden) equilibrated with 25 mM Bis-Tris buffer, pH 6.2, and bound protein was eluted with a linear 0−0.5 M NaCl gradient. The purified recombinant protein gave a single band on a SDS-PAGE gel, and the concentration of the E115S mutant protein was determined by measuring the absorbance at 280 nm ($\varepsilon_{280} = 64\ \mathrm{mM^{-1}\ cm^{-1}} = 1.888\ \mathrm{g^{-1}\ mL^{-1}}$).[29]

4.2. Lam16A Crystallization and Ligand Soaking. 8 mg/mL of the Lam16A E115S variant in 10 mM sodium acetate was mixed with an equal volume of crystallization buffer consisting of 20% (w/v) PEG 3350, 0.2 M ammonium nitrate, and 10 mM sodium acetate buffer, pH 5.0, at 20 °C. Crystals were obtained by the hanging-drop vapor diffusion method,[30] with an initial drop size of 2 μL.

The Lam16 E115S-ligand complexes were obtained by soaking isolated apo-crystals for 24 h in cryoprotectant with an additional 5 mM laminarioligosaccharides αL7F (synthesis description, *KHM Jonsson et al., in preparation*) or laminariheptasaccharide (DP 7) (Seikagaku Corporation, Tokyo, Japan). Cryoprotectant contained 35% (w/v) PEG 3350, 0.2 M ammonium nitrate, and 10 mM sodium acetate buffer, pH 5.0

4.3. Glycosynthase Reaction and Detection of Products. 20 mM αL7F in water was pipetted into Eppendorf tubes. The Lam16A E115S concentration was measured at 280 nm and diluted to 2 μM in 0.2 M sodium phosphate buffer (pH 7). The enzyme solution was added to an equal amount of substrate solution (total volume 150 μL). The reaction mixture was incubated in a 30 °C water bath, and 15 μL of sample were extracted at 10 min, 8 h, 24 h, and 48 h and frozen at −20 °C. A simultaneous negative control with buffer instead of enzyme was also performed. Samples were thawed for 2 min, and 15 μL of water were added before measuring.

The samples were analyzed by reversed-phase chromatography (RPC) on an Agilent 1100 HPLC system using a Reprosil-Pur C18-AQ.5um 100 mm × 4.6 mm column (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany). 20 μL of sample were manually injected at the start of a 30 min gradient program: 24 min 0−20% MeCN, 1 min to 95% MeCN, 1 min at 95% MeCN, 4 min at 0% MeCN. Eluted compounds were detected with a Corona charged aerosol detector (CAD; ESA Biosciences, Chelmsford, MA, USA) set at 500 pA.

To isolate the product for MS and NMR analyses, a 500 μL reaction was prepared and separated as above, after 24 h of incubation at room temperature. Five portions of 25 μL were injected to the column, and a manual splitter was used to diverge ∼20% of the outflow to the CAD detector and the rest to the fraction collector. The peak at the 17.7 min elution time was collected and stored at −20 °C.

4.4. Mass Spectrometry of Cyclic Heptaglucan Product. Mass spectra were obtained using a MALDI-TOF instrument (Ultraflex TOF/TOF, Bruker Daltonics, Bremen, Germany). The instrument was calibrated with a mixture of peptides (Peptide calibration standard II, Bruker Daltonics) and operated in reflector mode with α-cyano-4-hydroxycinnamic acid as the matrix.

4.5. NMR Spectroscopy. ^{1}H and ^{1}H,^{13}C-HSQC NMR spectra of cL7 (1 mM) in D_2O were recorded at 70 °C on a Bruker AVANCE II 500 MHz spectrometer equipped with a 5 mm probe (Bruker Biospin). Data processing was performed using vendor-supplied software. Chemical shifts are reported in ppm using external sodium 3-methylsilyl-(2,2,3,3-$^2H_4$)-propanoate (TSP, δ_H 0.00) or 1,4-dioxane in D_2O (δ_C 67.40) as references.

The translational diffusion measurement[17] was performed on a Bruker Avance III 600 MHz spectrometer at 70 °C by a stimulated-echo NMR experiment with bipolar gradient pulses. The duration of the gradient pulses (δ) was set to 2 ms, and the diffusion period (Δ) to 100 ms. The PFG strength was varied from 2% to 90% of 56 G cm^{-1} over 32 steps. The spectra were recorded with 16k data points, and the FIDs were processed in a DOSY mode resulting in a 2D plot with the ^{1}H chemical shift on the *x*-axis and the logarithm of the translational diffusion coefficient on the *y*-axis.

4.6. Molecular Dynamics Simulations. The starting coordinates of L7 were taken from the crystal structure, and cL7 was built from L7 by connecting the end residues. The free energy conformational maps were derived from population analysis of the glycosidic torsions as described previously.[18] The MM3 force field as implemented in the TINKER suite (dasher.wustl.edu/tinker/) and a dielectric constant of 4 were used to calculate the trajectories for L7 and cL7 at 300 K. The length of the MD simulation was 100 ns with sampling every 0.5 ps. The Conformational Analysis Tools (CAT) software (www.md-simulations.de/CAT/) was used for data processing and analysis.

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Supporting Information Available: Three files of raw data for the RPC-HPLC chromatograms shown in Figure 3 and one for the MALDI-TOF mass spectrum shown in Figure 4. Each file consists of two space-delimited columns with the detector *x* and *y* output value pairs. The RPC-HPLC chromatograms (ja909129b_si_001.txt, ja909129b_si_002.txt, ja909129b_

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si_003.txt) were run after 0, 4, and 48 h, respectively, of glycosynthase reaction, and the files contain the recorded output signal from the Corona CAD set at 500 pA full scale deflection (1000 mV). *x*, elution time in minutes, from 10 to 20 min; *y*, detector signal in mV. The ja909129b_si_004.txt file contains the raw data from the MALDI-TOF analysis of the glycosynthase reaction product eluting at 17.7 min in the HPLC chromatograms. *x*, mass/charge ratio, from 500 to 5000 *m*/*z*; *y*, detector output signal in arbitrary units. This material is available free of charge via the Internet at http://pubs.acs.org.

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