Structure of a PL17 Family Alginate Lyase Demonstrates Functional Similarities among Exotype Depolymerases

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**Background:** Exotype polysaccharide lyases can cleave glycosidic bonds to catabolize complex carbohydrates into simple monosaccharides.

**Results:** The first characterization of a family 17 polysaccharide lyase (PL17) provides insights into the mechanism of alginate depolymerization.

**Conclusion:** The structure of a PL17 enzyme illustrates unexpected similarities to other polysaccharide lyases despite a lack of sequence conservation.

**Significance:** These studies demonstrate an unexpected evolutionary relationship among the polysaccharide lyases.

Brown macroalgae represent an ideal source for complex polysaccharides that can be utilized as precursors for cellulosic biofuels. The lack of recalcitrant lignin components in macroalgal polysaccharide reserves provides a facile route for depolymerization of constituent polysaccharides into simple monosaccharides. The most abundant sugars in macroalgae are alginate, mannitol, and glucan, and although several classes of enzymes that can catabolize the latter two have been characterized, studies of alginate-depolymerizing enzymes have lagged. Here, we present several crystal structures of Alg17c from marine bacterium *Saccharophagus degradans* along with structure-function characterization of active site residues that are suggested to be involved in the exolytic mechanism of alginate depolymerization. This represents the first structural and biochemical characterization of a family 17 polysaccharide lyase enzyme. Despite the lack of appreciable sequence conservation, the structure and β-elimination mechanism for glycolytic bond cleavage by Alg17c are similar to those observed for family 15 polysaccharide lyases and other lyases. This work illuminates the evolutionary relationships among enzymes within this unexplored class of polysaccharide lyases and reinforces the notion of a structure-based hierarchy in the classification of these enzymes.

The increasing cost and limited reserve of fossil fuels have resulted in massive efforts in fields of research aimed at the discovery of efficient sources of renewable energy (1). Over the past decade, massive resources have been invested in the optimization of methods for extracting ethanolic precursors from terrestrial biomass, such as corn and perennial grasses (2). Comparatively, less effort has been directed toward the bioprocessing of marine algal biomass, which has shown potential as a replacement for land-based plant materials (3). Red, brown, and green algae contain an abundance of polysaccharide-derived sugars and, because of tolerance of changes in climate or soil conditions, represent the most sustainable source for fermentative biofuels (4). Moreover, large scale cultivation of algae can be carried out in controlled environments, such as photo bioreactors. The unique carbohydrate composition of algae presents a formative challenge for bioprocessing (5). As a consequence, there has been increasing interest in the discovery and development of enzymes that can depolymerize algal polysaccharides into fermentable monomeric sugars.

The most widely distributed component of brown algae is alginate, a cell wall polysaccharide composed of polymeric blocks of α-(1,4) O-linked β-D-mannuronate (M) and its C5 epimer α-L-guluronate (G). These monosaccharides can form homopolymeric blocks of G residues (G blocks) or M residues (M blocks) or heteropolymers of alternating M and G (MG blocks). Depolymerization of alginate is catalyzed by diverse alginate lyases, which target the 1→4 O-linkage via β-elimination of the 4-O-glycosidic bond (6). Endotype alginate lyases, such as A1-I, A1-II, and A1-III from *Sphingomonas* sp. A1, depolymerize alginate into di-, tri-, and tetrasaccharides, which are then further processed by an exotype alginate lyase to yield monosaccharides that further undergo non-enzymatic conversion into 4-deoxy-1-erythro-5-hexoseulose uronic acid (Fig. 1). Within the Carbohydrate-Active EnZymes (CAZy) database (7), alginate lyases are categorized into one of 21 different polysaccharide lyase (PL) families, and exotype activity has been experimentally determined for the PL15 and PL17 families of enzymes (8). However, there is limited conservation of primary sequence between PL15 and PL17 family enzymes with less than 15% sequence identity between representative family members. In addition, PL17 enzymes lack nearly all the requisite catalytic and functionally critical residues identified through structural and biochemical studies of the PL15 enzyme Atu3025 from *Agrobacterium tumefaciens* (9). To date, there is replacement for land-based plant materials (3). Red, brown, and green algae contain an abundance of polysaccharide-derived sugars and, because of tolerance of changes in climate or soil conditions, represent the most sustainable source for fermentative biofuels (4). Moreover, large scale cultivation of algae can be carried out in controlled environments, such as photo bioreactors. The unique carbohydrate composition of algae presents a formative challenge for bioprocessing (5). As a consequence, there has been increasing interest in the discovery and development of enzymes that can depolymerize algal polysaccharides into fermentable monomeric sugars.

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Crystal Structure of PL17 Alginate Lyase

The marine bacterium *Saccharophagus degradans* 2-40 has been shown to degrade several complex polysaccharides, including alginate, and bioinformatics analysis of its genome identifies nine putative alginate lyases belonging to PL families 7, 14, 17, and 18 (10). Biochemical characterization of the *S. degradans* PL17 enzyme (Alg17c) demonstrates that this exo-type enzyme can depolymerize alginate di-, tri-, and tetrasaccharides into monosaccharides, providing the necessary precursor for ethanol fermentation (11). However, given the lack of sequence similarity with PL15 and lack of conservation of active site features, little is known about the mechanism of any PL17 family member.

EXPERIMENTAL PROCEDURES

Materials—*S. degradans* 2-40 genomic DNA was purchased from the American Type Culture Collection. Low viscosity alginate was purchased from MP Biomedicals. Protein molecular weight markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad. All
other reagents were purchased from Fisher Scientific and were of the highest purity available.

Preparation of Alginate Oligosaccharide Substrate—Alginate oligosaccharides were generated by enzymatic depolymerization of low viscosity alginate using alginate lyase from Flavobacterium multivorum (Sigma CAS 40591-57-9). Briefly, 150 μl of enzyme at a concentration of 1 unit/ml was added to a solution containing 0.1% sodium alginate at pH 6.3, and the mixture was incubated at 37 °C for 30 min. Reaction mixtures containing up to 20 mg of digested polysaccharides were loaded onto a DEAE-Sephadex A-25 column (2.8 x 50 cm) on an Agilent 1100 Series HPLC system. The solvent flow rate was 0.3 ml/min with a temperature of 65 °C. The products were eluted using the following gradient where solvent A is 0.2% TFA in water and solvent B is methanol: 30% B from 0 to 10 min and gradient from 30 to 90% B from 10 to 40 min. The final products were lyophilized, and masses for the lyophilized products were determined using an Agilent 1100 LC/MSD Trap XCT Plus mass spectrometer (Agilent Technologies, Inc., Palo Alto, CA).

Cloning and Site-directed Mutagenesis—Amplification of Alg17c (GenBank™ accession number ABDB2539.1) from S. degradans genomic DNA was done using PCR, and the resultant insert was cloned into pET-28b. To facilitate heterologous expression, the expression construct lacks the first 24 residues that encode the signal peptide. Site-specific mutations in the expression construct were done by Site-directed mutagenesis according to the manufacturer’s protocol (Phusion Site-directed Mutagenesis kit, Agilent Technologies, Inc., Santa Clara, CA). The PCR product (12.5 μl) was digested with methylation-dependent restriction enzyme DpnI at 37 °C for 8 h to digest the parental plasmid DNA. Escherichia coli DH-5a competent cells were transformed with the product from the DpnI restriction digestion and plated onto lysozyme broth (LB) solidified with Bacto agar (Difco) containing 25 μg/ml kanamycin sodium salt, and the plates were incubated at 37 °C overnight. Individual colonies were cultivated in LB medium supplemented with kanamycin until stationary phase was reached, and plasmids were extracted using a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). The integrity of all of the constructs was confirmed by DNA sequencing (W. M. Keck Center for Comparative and Functional Genomics, University of Illinois).

Recombinant Protein Production and Purification—E. coli BL21-(DE3)-Rosetta cells harboring a recombinant plasmid bearing either the Alg17c gene or variants were cultured in LB containing 25 μg/ml kanamycin for ~12 h at 37 °C with vigorous shaking. Gene expression was induced when the absorbance at 600 nm reached ~0.5 with the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, and the temperature was decreased to 16 °C followed by incubation for an additional 16 h at 200 rpm. Cell pellets were harvested from the cultures by centrifugation at 5,000 x g for 30 min at 4 °C, washed with 10 ml of buffer (50 mM Tris, 300 mM KCl, pH 7.5), and frozen as cell pellets at ~80 °C. Preparation of selenomethionine (SeMet)-labeled Alg17c was carried out by repression of methionine synthesis in defined media (12).

Frozen cell pellets were thawed, resuspended in 35 ml of ice-cold binding buffer, and passed through an Emulsiflex C-3 homogenizer (Avestin, Ottawa, Canada) to rupture the cells. The cell lysates were clarified by centrifugation at 16,000 x g for 30 min at 4 °C, and the supernatant containing the soluble fraction was loaded onto a 5-ml immobilized metal ion affinity resin column (Hi-Trap nickel-nitritolatriaetate acid, GE Healthcare) pre-equilibrated with binding buffer (50 mM Tris-HCl, 300 mM KCl, 20 mM imidazole, pH 7.5). The column was extensively washed with lysis buffer supplemented with 30 mM imidazole and eluted by a linear gradient to 200 mM imidazole. The polyhistidine affinity tag was removed by overnight incubation with thrombin (1 unit/mg of protein). Samples were concentrated using a 10,000 molecular weight cutoff Amicon centrifugal filters and further purified using size exclusion chromatography (Superdex HiLoad™ 75 16/60, GE Healthcare) in 20 mM HEPES, pH 7.5, 100 mM KCl buffer.

Crystallization and Data Collection—Crystals of wild-type, SeMet-labeled, and variant Alg17c were grown by the hanging drop method. 15 μg/ml protein was incubated in a condition containing 0.1 M Tris, pH 8.0, 5% 2-methyl-2,4-pentanediol, 10% polyethylene glycol (PEG) 6000 over the course of 3 days at 16 °C. Crystals were soaked in a solution containing 20% PEG 6000, 12% ethylene glycol, 0.1 M Tris, pH 8.0, 5% 2-methyl-2,4-pentanediol and flash frozen in liquid nitrogen.

Phasing and Structure Determination—Flash cooled crystals of wild-type Alg17c diffraction x-rays to 1.85-Å resolution using a Mar 300 charge-coupled device detector at an insertion device synchrotron beam line (LS-CAT Sector 21 ID-G, Advanced Photon Source, Argonne, IL). These crystals belong to space group P2_1 with unit cell parameters a = 85.5 Å, b = 126.4 Å, c = 87.6 Å, and β = 111.5° with two molecules in the crystallographic asymmetric unit. A 3-fold redundant data set was collected to 1.85-Å resolution with an overall Rmerge of 9.2% and I/σ(I) of 2.1 in the highest resolution shell. All data were indexed and scaled using either the HKL2000 (13) package or XDS (14). Crystallographic phases were determined using single wavelength anomalous diffraction data collected from a crystal of SeMet-labeled protein. Diffraction data were collected to 2.2-Å resolution and processed as described above. Heavy atom sites were determined using HySS and imported into SHARP for maximum likelihood refinement (15). The resultant phases were further improved by 2-fold non-crystallographic symmetry averaging and subjected to automated model building as implemented in ARP/wARP (16). The model was fitted using Coot (17) and further improved by rounds of refinement with REFMAC (18). Cross-validation was routinely used throughout the course of model building and refinement using 5% of the data in the calculation of the free R-factor (19).

The structure of Alg17c variants H202L (1.7-Å resolution) and Y258A (2.45-Å resolution) and the co-crystal structure of Y258A in complex with the alginate trisaccharide (1.9-Å resolution) were determined by molecular replacement using the coordinates of unliganded, wild-type Alg17c as a search probe.
Table 1. Data collection, phasing, and refinement statistics

| Data collection       | Native H202L | Y258A | Y258A ΔMMG |
|-----------------------|--------------|-------|------------|
| a, b, c (Å)           | 85.5, 126.4, 87.6 | 85.5, 126.4, 87.6 | 85.5, 126.4, 87.6 |
| Resolution (Å)        | 25.0–1.85 | 25.0–1.85 | 25.0–1.85 |
| Total reflections      | 470,419 | 243,785 | 378,202 |
| Unique reflections    | 144,904 | 98,566 | 191,532 |
| Completeness (%)      | 92.6 (2.2) | 6.8 (67.3) | 5.4 (55.0) |
| Redundancy            | 3.3 (3.3) | 4.3 (4.3) | 4.1 (3.7) |

Each of the structures was refined and validated using the procedures detailed above. For each of the structures, the stereochemistry of the model was monitored throughout the course of refinement using PROCHECK (20) and MolProbity (21). Relevant data collection and refinement statistics are provided in Table 1. The refined atomic coordinates have been deposited in the Protein Data Bank (22).

Kinetic Analysis of Wild-type Alg17c—Kinetic characterization was carried out on wild-type Alg17c as well as the variants H202L and Y258A using low viscosity alginate as the substrate. For each assay, 0.613 μM enzyme was added to 1 ml of mixture containing 50 mM Tris-HCl, pH 7.5 and varying concentrations of substrate (0.1–10 mg/ml). The mixture was incubated at 30 °C for 1 h and then boiled for 5 min to stop the reaction, and the thiobarbituric acid assay method was used to quantify the amount of unsaturated uronic acids produced. Briefly, 40 μl of the reaction mixture was added to 0.25 ml of 1 M H5IO6 (in 50 mM H2SO4) and incubated at room temperature for 20 min followed by the addition of 0.50 ml of 2% sodium arsenite to the mixture and incubation for 2 min with vigorous shaking. 2 ml of 0.3% thiobarbituric acid at pH 2 was then added, and the mixture was heated to 100 °C for 10 min. After cooling, the resultant condensation of β-formylpyruvic acid with thiobarbituric acid was monitored by measuring the absorbance at 548 nm. 1 unit of enzyme activity was defined as the amount required to liberate 1 μM β-formylpyruvic acid/min.

Thin Layer Chromatographic (TLC) Analysis—Alginate oligosaccharides with degrees of polymerization of 2, 3, and 4 (0.5 mg/ml) were incubated with 0.6 μM Alg17c in a buffer of 50 mM Tris-HCl, pH 7.5 at 30 °C for 1 h. Reaction products were analyzed by TLC using a solvent of n-butyl alcohol:formic acid:water (4:6:1, v/v/v). The depolymerized products were visualized by developing the plates with 10% (v/v) sulfuric acid in ethanol followed by incubation at 100 °C for 20 min. Standards consisted of either D-glucose (2 mM) monosaccharide or D-trehalose (2 mM) disaccharide.

Isothermal Titration Calorimetry—The MicroCal VP-ITC system from GE Healthcare was used to calculate dissociation constants for the binding of oligosaccharide to catalytically inactive Alg17c. Purified enzyme was concentrated to 15 mg/ml and diluted in 20 mM HEPES, pH 7.5, 100 mM KCl buffer. Oligosaccharide with a degree of polymerization between 2 and 4 was diluted in the same buffer to ~1 mM. Both Alg17c and substrate were degassed and brought to room temperature. The reference cell contained deionized water, and the sample chamber was washed with deionized water and methanol before the sample was injected. The protein sample (in a 1.44-ml reaction cell) was injected with 26 successive 10-μl aliquots of ligand at 300-s intervals. Data were fitted by nonlinear regression using a single site model (MicroCal Origin).

RESULTS AND DISCUSSION

Kinetic Analysis of Wild-type Alg17c—To facilitate heterologous expression, the first 24 residues encoding the signal peptide were not included for any of the expression constructs. Determination of the kinetic parameters for degradation of low
viscosity alginate by Alg17c was carried out using the thiobarbituric acid assay method (Table 2). Wild-type Alg17c was shown to have a $k_{\text{cat}}$ of 56.9 s$^{-1}$, $k_{\text{cat}}/K_m$ of 2.6 $\times 10^6$ M$^{-1}$ s$^{-1}$, $K_m$ of 22.3 $\mu$M, and $V_{\text{max}}$ of 34.9 $\mu$M s$^{-1}$. These parameters are well within the range of values for enzymes from other polysaccharide lyase classes and are also similar to values reported for other alginate lyases, including AlgI, from Pseudomonas aeruginosa (23) and Atu3025 from A. tumefaciens (9). Kinetic parameters for wild-type and site-specific active site variants of Alg17c are reported in Table 2 and discussed in the relevant sections below.

**Determination of Polymer Specificity of Alg17c**—The minimal polymer length that can be processed by Alg17c was determined by thin layer chromatographic analysis of Alg17c reaction products using alginate oligosaccharides of defined length. Treatment of a mixture containing oligosaccharides with degrees of polymerization of 2, 3, and 4 resulted in complete processing only of the tri- and tetrasaccharide substrates. In contrast, the disaccharide substrate was only partially processed. These results are consistent with Alg17c having a minimal polymer specificity of 3. Kinetic analysis of wild-type Alg17c using a trisaccharide substrate yielded a $k_{\text{cat}}$ of 62.4 s$^{-1}$, $k_{\text{cat}}/K_m$ of 8.2 $\times 10^6$ M$^{-1}$ s$^{-1}$, $K_m$ of 7.7 $\mu$M, and $V_{\text{max}}$ of 38.2 $\mu$M s$^{-1}$. These kinetic values are similar to those determined using heteropolymeric low viscosity alginate as a substrate, consistent with the notion that an alginate trisaccharide represents the minimal length substrate for Alg17c.

**Overall Structure of Alg17c**—The structure of wild-type Alg17c was determined to 1.85 Å resolution using phases determined by single wavelength anomalous diffraction measurements collected using crystals of SeMet-labeled protein (relevant data collection statistics are provided in Table 1). In each of the structures, no electron density can be observed for the first 6 residues at the amino terminus, and no effort was made to model these residues. The final model for each of the structures consists of all residues spanning His$^{30}$–Arg$^{734}$.

The overall architecture of each Alg17c monomer consists of two domains: an amino-terminal imperfect $\alpha$-barrel (consisting of residues His$^{30}$–Glu$^{665}$) and the carboxy-terminal $\beta$-sheet domain (composed of residues Pro$^{770}$–Arg$^{735}$) (Figs. 2, A and B). There are 13 helices in the amino-terminal domain, resulting in a topological arrangement that is similar to an $\alpha_6/\alpha_6'$-barrel but with an extra helix that buttresses against the side of the barrel. The arrangement of the $\alpha_6/\alpha_6'$-helices produces an open barrel structure, and this additional helix serves to maintain rigidity of the barrel. The carboxy-terminal domain is composed of three co-planar layers of antiparallel $\beta$-strands arranged as sheets and further supported by four small helices near the top layer that interject between the two domains (Figs. 2, A and B). Sequence conservation among other PL17 family members extends throughout all of the secondary structural elements (Fig. 2C), suggesting that the Alg17c structure is representative of all PL17 enzymes. In contrast, all of the alginate lyases in nearly all other PL families adopt either an $\alpha$-barrel or a jelly roll fold (8), which is distinct from the combination of the $\alpha_6/\alpha_6'$-barrel and antiparallel $\beta$-sheet arrangement observed in the PL17 structure.

The general domain structure observed in Alg17c is reminiscent of that observed in the structure of the PL15 family enzyme Atu3205 from A. tumefaciens (Protein Data Bank code 3AOO) (9). Although the two enzymes are topologically distinct as evidenced by an r.m.s.d. value of 3.4 Å upon least square alignment of 567 Cα atoms, the PL15 family Atu3205 also consists of an $\alpha_6/\alpha_6'$-barrel joined to a carboxy-terminal antiparallel $\beta$-sheet. A recent structure–based classification of polysaccharide lyases terms this topological class as that of a multidomain (αα)n toroid (Fig. 3) (24). A DALI search (25) against the Protein Data Bank identifies Atu3205 (Z-score of 27.1, r.m.s.d. of 5.7 Å over 562 aligned Cα atoms, r.m.s.d. of 3.5 Å over 297 aligned Cα atoms from the amino-terminal domain, and r.m.s.d. of 2.8 Å over 271 aligned Cα atoms from the carboxy-terminal domain) as the closest structural homolog (Fig. 3B) along with type II heparinase from Pedobacter heparinus (Protein Data Bank code 2FUQ; Z-score of 27.4, r.m.s.d. of 4.1 Å over 557 aligned Cα atoms, r.m.s.d. of 3.8 Å over 285 aligned Cα atoms from the amino-terminal domain, and r.m.s.d. of 3.0 Å over 280 aligned Cα atoms from the carboxy-terminal domain) (Fig. 3C) (26) and type III heparinase from Bacteroides thetaiotamicron (Protein Data Bank code 4FNV; Z-score of 26.8, r.m.s.d. of 4.5 Å over 529 aligned Cα atoms, r.m.s.d. of 4.0 Å over 270 aligned Cα atoms from the amino-terminal domain, and r.m.s.d. of 3.0 Å over 257 aligned Cα atoms from the amino-terminal domain) (27). More distant homology is observed with hyaluronate lyase (Protein Data Bank code 1LXK; Z-score of 18.0, r.m.s.d. of 5.8 Å over 562 aligned Cα atoms, r.m.s.d. of 3.5 Å over 294 aligned Cα atoms from the amino-terminal domain, and r.m.s.d. of 3.8 Å over 266 aligned Cα atoms from the carboxy-terminal domain) (Fig. 3D) (28) and xanthan lyase (Protein Data Bank code 1X11; Z-score of 17.3, r.m.s.d. of 5.7 Å over 549 aligned Cα atoms, r.m.s.d. of 3.4 Å over 288 aligned Cα atoms from the amino-terminal domain, and r.m.s.d. of 3.9 Å over 258 aligned Cα atoms from the amino-terminal domain) (29). Each of these enzymes is composed of an $\alpha$-barrel domain fused to a domain consisting of $\beta$-strands, and this structural superfamily has been designated as the multidomain (αα)n
Crystal Structure of PL17 Alginate Lyase

A (α/α) barrel

β sandwich

Leader sequence

Saccharophagus-Alg17
Sphingomonas-Alg1
Pseudomon-Al2
Saccharophagus-Alg17
Sphingomonas-Alg1
Pseudomon-Al2
Saccharophagus-Alg17
Sphingomonas-Alg1
Pseudomon-Al2
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Sphingomonas-Alg1
Pseudomon-Al2
Saccharophagus-Alg17
Sphingomonas-Alg1
Pseudomon-Al2

Zn$^{2+}$
Crystal Structure of PL17 Alginate Lyase

**FIGURE 3. Conservation of structure among enzymatically diverse polysaccharide lyases.** A comparison of the crystal structures of Alg17c (colored in pink and green) (A) with Atu3205 from A. tumefaciens (in blue and brown; Protein Data Bank code 3A0O) (B), heparinase II form P. heparinus (in umber and lavender with the zinc ion in red; Protein Data Bank code 2FUQ) (C), and hyaluronate lyase from Streptococcus pneumonia (in cyan and tan; Protein Data Bank code 1LXK) (D) is shown.

...toroid structural class (24). Although the helical dispositions are quite distinct, the α6/α6-barrel domain of heparinase II also contains an additional helix. The structural homology to PL15 enzymes and to other multidomain (α/α)n-toroid class enzymes is especially notable due to the fact that there is no significant conservation between the primary sequence of Alg17c and any of its structural homologs.

Given the similar exolytic mode of action on alginate substrates, the structural homology between the PL17 family Alg17c and the PL15 family Atu3205 is noteworthy. The PL15 enzyme contains an additional amino-terminal domain of roughly 100 residues composed of seven β-strands but otherwise shares similar structural features (9). This conservation is unexpected given the low 13% sequence identity shared between the two enzymes. Given the lack of sequence conservation, it is not surprising that the α6 active site of Alg17c is quite distinct from that of Atu3205. Nearly all of the residues that have been demonstrated to play a critical role in alginate depolymerization by Atu3205 are divergent in Alg17c, and the steric confines of the two active sites are also different. A thorough description of these differences is detailed under “Co-crystal Structure of an Alg17c Variant in Complex with an Alginate Trisaccharide” (see below).

**Oligomerization State of Alg17c—**Analytical size exclusion chromatographic studies are consistent with the formation of Alg17c homodimers in solution (data not shown), and this dimer is recapitulated in the slightly different crystal forms of wild-type and variant Alg17c (Fig. 2B). Dimerization results in the burial of roughly 1300 Å² of surface area per monomer (measured using a probe radius of 1.4 Å). Formation of the homodimer results in the insertion of an antiparallel β-turn, encompassing residues Gly660–Ala672, from the carboxyl-terminal β-sheet domain of one monomer into the α6/α6-barrel of the other monomer (Fig. 2B). Alg17c elutes as a dimer in analytical size exclusion experiments even at low concentrations near the limit of detection. Given the experimental data and the observation of intersubunit interactions in the Alg17c crystal structure, it is likely that the homodimer represents the biologically relevant form of the enzyme.

**A Zinc-binding Site in Alg17c—**Experimental electron density maps from data collected on crystals of SeMet-labeled Alg17c near the selenium absorption edge (λ = 0.9786) showed an electron-density feature near the vicinity of the amino- and carboxyl-terminal interface. The side chains of His415, Asp433, and His464 along with three solvent molecules are located around this density with the nitrogen and oxygen atoms engaging in near octahedral coordination geometry (Fig. 4). Given the coordination distance and geometry, this peak has been tentatively assigned to a bound Zn²⁺ ion. This electron density is enhanced in a data set that was collected at λ = 1.0, which would correspond to an f' value of 2.55 at this wavelength for zinc. A similar peak was also observed in the structure of P. heparinus heparinase II at nearly the same location and was also modeled as a bound zinc ion (26) (Fig. 3C). Coordination of the metal joins the small helices located at the juncture of the two domains with the top β-sheet layer of the carboxyl-terminal β-strand domain. As in heparinase II, the metal ion is located over 15 Å away from the active site and likely only plays a structural role. Specifically, His415 and Asp433 are both part of different loop regions that include residues that may function in binding to substrate oligosaccharides. Presumably, the metal ion establishes the orientation of His415 and Arg438, which would otherwise be mobile, to set these residues for interactions with substrate.
Crystal Structure of PL17 Alginate Lyase

To probe the relevance of this zinc ion in catalytic activity, one of the metal-binding protein ligands, His415, was replaced, and the H415A mutant was kinetically characterized. The catalytic activity of this mutant was slightly compromised compared with the wild type with a negative effect on $k_{cat}/K_m$ ($2.6 \times 10^3 \text{M}^{-1} \text{s}^{-1}$, which is nearly a 1,000-fold lower than that of the wild type). These results are consistent with the proposed role of the metal ion in establishing the orientation of residues that may be important for engaging oligosaccharide substrates.

Despite the structural similarities of Alg17c and the PL15 family of enzymes, no metal ion is observed at a similar location in the structure of Atu3205 (9). Moreover, only one of the three metal coordination residues is conserved (His533 in place of His415), and the other Asp433 and His464 are Gln551 and Lys573, respectively. The cavity created by the lack of a metal ion in Atu3205 is partially compensated by the insertion of a hydrophobic Phe414 into the putative binding site. The presence of a bound metal is likely a distinguishing feature of PL17 family of enzymes as equivalent metal coordination residues are also observed in the sequences of other PL17 members, such as AlyIII (from Pseudomonas sp. OS-ALG9) (30) and AlgL (from Sphingomonas sp. MJ3) (31) that have been partially characterized (Fig. 2C).

**FIGURE 4. Difference Fourier maps of the bound metal ion.** A stereoview of the Alg17c metal-binding site derived from the refined coordinates of Alg17c is shown. The Alg17c carbon atoms are shown in yellow ball-and-stick representation, metal-bound solvent molecules are colored in black, and the metal is shown in red. Superimposed is a difference Fourier electron density map contoured at 5σ over background in blue and 15σ in red calculated with coefficients $|F_{obs}| - |F_{calc}|$. A structure-based alignment identifies His202 and Tyr258 in Alg17c situated at equivalent positions to His311 and Tyr365 in Atu3205. Based on this alignment, the H202L and Y258A mutations were generated in Alg17c for co-crystallization studies with intact substrate oligosaccharides. To determine whether Tyr258 and His202 are indeed the catalytic acid and base, respectively, in Alg17c, the Y258A and H202L variants were subject to kinetic characterization. Although the Y258A variant was devoid of any activity, the H202L variant has a $k_{cat}$ of 10.9 s$^{-1}$ and $K_m$ of 92 μM. Consequently, although the H202L variant is compromised in activity, it is still active, and therefore, His202 is not likely to participate in acid/base chemistry. However, as the H202L Alg17c yielded crystals that diffracted to a slightly higher resolution than the wild type, further structural characterization of this variant was carried out.

To ensure that the mutations had not resulted in significant changes to the enzyme, the structure of H202L Alg17c and the structure of Y258A Alg17c were determined to 1.7- and 2.45-Å resolution, respectively. The structures of the mutant enzymes were essentially identical to that of the wild-type enzyme with all of the differences constrained to minor movements of loop regions in the carboxyl-terminal β-sheet domain.

Small oligosaccharide substrates were generated from crude alginate polymer and purified using size exclusion chromatography for biochemical studies. To determine the affinity of Alg17c for alginate substrates, isothermal titration calorimetry was carried out using the Y258A variant. This variant binds to the trisaccharide ligand with 1:1 stoichiometry with an association affinity of $9.9 \times 10^3 \text{M}^{-1}$ and is largely enthalpically driven (Fig. 5).

**FIGURE 5. Isothermal titration calorimetric analysis.** Binding isotherms characterizing the interaction between a ΔMMG trisaccharide with the Y258A variant of Alg17c are shown. Data were fit to a single site model. deg, degrees.

Co-crystal Structure of an Alg17c Variant in Complex with an Alginate Trisaccharide—The nomenclature for substrate binding in lyase active sites defines subsites that engage each saccharide with cleavage occurring at the glycosidic bond that links the sugars at subsites $-1$ and $+1$. In the canonical exolytic mechanism, the negative charge on the C6 carboxylate of the saccharide at subsite $+1$ is stabilized, facilitating the removal of the C5 proton by a general base and subsequent donation of a proton to the O4 atom by a general acid (32). Structural and biochemical studies on the PL15 family Atu3205 suggest that His311 and Tyr365 function as the catalytic base and acid, respectively. A structure-based alignment identifies His202 and Tyr258 in Alg17c situated at equivalent positions to His311 and Tyr365 in Atu3205. Based on this alignment, the H202L and Y258A mutations were generated in Alg17c for co-crystallization studies with intact substrate oligosaccharides. To determine whether Tyr258 and His202 are indeed the catalytic acid and base, respectively, in Alg17c, the Y258A and H202L variants were subject to kinetic characterization. Although the Y258A variant was devoid of any activity, the H202L variant has a $k_{cat}$ of 10.9 s$^{-1}$ and $K_m$ of 92 μM. Consequently, although the H202L variant is compromised in activity, it is still active, and therefore, His202 is not likely to participate in acid/base chemistry. However, as the H202L Alg17c yielded crystals that diffracted to a slightly higher resolution than the wild type, further structural characterization of this variant was carried out.

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To further define active site residues that may participate in both substrate recognition and the exolytic mechanism, small oligosaccharide substrates with a degree of polymerization between 2 and 4 were generated and purified from crude alginate polymer and then used to soak into crystals of Y258A Alg17c. Co-crystals of Y258A Alg17c diffracted to 1.9-Å resolution. Although molecular replacement using the coordinates of the unliganded wild-type Alg17c gave a satisfactory solution, the resultant model could not be refined to any acceptable criteria. Inspection of the resultant electron density maps revealed significant conformational changes at the carboxyl-terminal β-sheet domain, and manual rebuilding of the region followed by crystallographic refinement resulted in a major decrease in the free R-value to below 0.3. At this point, clear and continuous electron density for the bound oligosaccharide could be observed in the vicinity of the putative active site residue, Tyr258. The high resolution of the data and the high quality of the corresponding electron density map reveals the bound trisaccharide as ΔMMG where ΔM denotes unsaturated D-mannuronate and M and G denote saturated D-mannuronate and L-glucuronate, respectively (Fig. 6). Following refinement of the model, the bound trisaccharide has crystallographic B-values (a relative measure of mobility) that are similar to those of the protein main chain, consistent with well ordered binding of the ligand (Table 1).

A superposition of the structures of unliganded and trisaccharide-bound structures of Alg17c reveals that the enzyme undergoes conformational changes upon binding of substrate (Fig. 6B). Specifically, an alignment of the ΔM barrel domains demonstrates that the carboxyl-terminal β-sheet domain undergoes a rigid body rotation of roughly 12° upon ligand binding that results in the displacement of various active site residues to be poised for interactions with the bound trisaccharide. This domain movement is necessary to position several critical active site residues in catalytically competent orientations (Fig. 6C).

A similar domain rotation movement has also been observed upon ligand binding by the PL15 family Atu3205 (in complex with ΔGGG) (9), and computational studies suggest that active site flexibility plays a significant role in substrate engagement in the PL8 hyaluronate lyase (28). Despite the fact that none of these three enzymes demonstrate any appreciable similarities in primary sequence, they share the common architecture of an α/α-barrel linked to a β-sheet domain, suggesting that conformational flexibility may be a common feature of all enzymes from this multidomain (α/α)_n toroid structural class.

FIGURE 6. Alg17c active site with bound trisaccharide. A, stereoview of the Alg17c active site derived from the co-crystal structure in complex with ΔMMG. The Alg17c carbon atoms are shown in yellow ball-and-stick representation, and the trisaccharide is shown in green. Superimposed is a difference Fourier electron density map (contoured at 2.7σ above background in blue and 6.0σ in red) calculated with coefficients |F_{obs} - F_{calc}| and phases from the final refined model with the coordinates of the trisaccharide deleted prior to one round of refinement. B, comparison of the overall structure of Alg17c in the absence (pink) and presence (cyan) of bound oligosaccharide substrate. C, close-up view of the Alg17c active site in the absence (pink) and presence (cyan) of substrate.
The bound ΔMMG trisaccharide occupies subsites −1, +1, and +2 with the nonreducing mannuronate occupying the deep cleft of the binding pocket where the pyranose ring stacks against the aromatic side chain of Tyr261. Hydrogen bond interactions that account for binding specificity include those from Asn146, Tyr257, Arg260, His413, Arg438, and Glu667. Additional polar residues engage the sugar indirectly through solvent-mediated interactions. At subsite +1, the saturated ω-mannuronate is engaged through a more limited set of hydrogen bond interactions with Gln146 and Asn201, indicative of flexibility in accommodating different bound saccharides at this position. The saturated α-glucuronate at the +2 position similarly interacts in a minimal manner with only enzyme residues Lys136 and Lys198 located within hydrogen bonding distance.

The co-crystal structure provides further insights into residues that likely function in acid/base chemistry and substrate stabilization for alginate depolymerization (Fig. 7). Assignment of the roles of functional residues is inferred based on their location in the proximity of the uronic acid carboxylate, the glycosidic bond, and the acidic C5 proton. In the co-crystal structure, the C6 carboxylate is located adjacent to Asn201 (3.2 Å from N82) and His202 (2.7 Å from Ne2), and these residues likely function to stabilize the carboxylate charge to lower the pKa of the C5 proton. Tyr450 is poised axially to the presumed location of the C5 proton (3.4 Å from Oη) where it likely functions as the general base. Lastly, Tyr450 is positioned in line with the glycosidic bond between subsites −1 and +1 (2.4 Å from Oη) and is likely the general acid that protonates O4 during bond cleavage.

Kinetic Characterization of Structure-based Active Site Mutants—To further confirm the presumed functions of the active site residues in catalysis, kinetic analyses were carried out on site-specific variants at each of the amino acids identified in the co-crystal structure. Consistent with the role of Tyr450 as the general base and Tyr258 as the general acid, the Y258A and Y450A single mutants were both completely devoid of activity. Parenthetically, we note that our assumption of Tyr258 as a participant in acid/base chemistry was correct; however, this residue is likely the general acid and not the general base as initially assumed. The Y261A mutant is also inactive, consistent with the proposed role for this residue in engaging the nonreducing mannuronate through stacking interactions. Based on the structure of the unliganded enzyme and structure-based alignments with Atu3025, His202 was presumed to be the general base. However, the H202L mutant was catalytically active (although compromised), and our co-crystal structure provides a rational for this observation. The function of His202 is not as a general base but rather to work in concert with Asn201 to stabilize the charge on the C6 carboxylate. Consequently, the H202L mutant should not result in a complete loss of activity, consistent with our kinetic analysis. Lastly, mutations at residues that stabilize the binding of the oligosaccharide (Asn149, Arg260, and His413) are significantly impaired with kcat/Km values that are orders of magnitude lower than that of the wild type. However, these variants still show some activity, reflective of the fact that the function of each of these residues is largely redundant.

Mechanistic Basis for the Exolytic Mode of Alginate Depolymerization by Alg17c—The mechanism for Alg17c likely follows the canonical mechanism for oligosaccharide lyase that necessitates active site residues that participate in acid/base chemistry and act to stabilize the negative charge on the C6 carboxylate (Fig. 7). The stepwise mechanism utilizes at subsite +1 a basic residue that lowers the pKa of the C5 proton to facilitate abstraction by a general base and a general acid to protonate the O4 atom during cleavage of the glycosidic bond. In many lyases, such as chondroitin AC lyase and hyaluronate lyase, the C6 carboxylate is not stabilized by an acidic residue but rather by an amide and/or His that forms a strong hydrogen bond with this moiety. The co-crystal structure of Alg17c reveals that Asn201 and His202 are near the vicinity of the C6 carboxylate and likely function in neutralizing the negative charge of the uronic acid carboxylate, and this was confirmed by mutational analysis at these residues. Tyr450 functions as the general base to abstract the C5 proton to facilitate glycosidic bond cleavage. Lastly, Tyr258 functions as the general acid to donate a proton to the O4 atom of the glycosidic bond during cleavage. The role of both of these residues is further borne out by our kinetic characterization of site-specific mutations.

The utilization of two tyrosine residues as both the general acid (Tyr258) and the general base (Tyr450) is unusual within the multidomain (α/α)n toroid lyase superfamily. Given that the phenolic side chain has a pKa of ~10, tyrosine is an atypical choice to function as a general base. For example, in the homologous PL15 family alginate lyase Atu3205, His311 functions as the catalytic base. Presumably, microenvironmental tuning of the pKa of both tyrosine residues can accommodate their function in acid/base chemistry. Analogously, a single tyrosine residue (Tyr234) functions as both the general acid and general base in chondroitin AC lyase (33).

Conclusions—Our studies provide the first crystallographic structures of a PL17 family enzyme, Alg17c from S. degradans. We also present the first detailed structure-function character-
organization of active site variants identified based on our co-crystal structures that establish the role of various residues in the chemistry of alginate depolymerization. Our combined results demonstrate a convergence both in structure and in function among the large class of lyases that fall within the multidomain (α/α), toroid structural class. Despite global similarities, numerous differences also exist, including the identity of the catalytic residues, and these cannot be identified by simple sequence or structure alignments. Additional studies of enzymes within this structural class will likely shed new insights into the mechanism of polysaccharide depolymerization.

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